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Rapid Communication

Isolation and Characterization of a cDNA Clone for a Harvest-Induced Asparagine Synthetase from *Asparagus officinalis* L.

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A full-length cDNA clone (pTIP27) encoding asparagine synthetase (AS; EC 6.3.5.4) was isolated from a cDNA library prepared from the tip section (apex to 30 mm) of *Asparagus officinalis* L. spears. The cDNA clone encodes an mRNA of 1978 bp, giving a derived protein of 66.5 kD molecular mass. The derived amino acid sequence is 81% homologous to AS from *Pisum sativum*. Only low levels of transcript for AS could be detected in growing spears, roots, or ferns. However, AS mRNA levels began to increase in the tips of harvested spears after 2 h at 20°C, and in the other sections of the spear after 4 h, suggesting that all sections of the spear were responding to the same postharvest signal. The results are discussed in relation to metabolic changes occurring in harvested spears.

Harvesting and handling of horticultural crops impose a series of stresses on the tissue, including wounding, dehydration, and separation from nutrient supply. Harvest stresses are particularly severe on organs that are actively growing at harvest, and, being unable to maintain metabolic homeostasis, these organs typically senesce rapidly (Huber, 1987). We are investigating the early physiological, biochemical, and molecular changes that follow harvesting of asparagus (*Asparagus officinalis*), with the aim of defining factors contributing to postharvest deterioration. The tip region of the spear, comprising immature, rapidly developing tissues, is particularly susceptible to harvest stress and is usually the first part of the spear to show symptoms of deterioration. Within 48 h of harvest, the respiration rate of tips declines markedly, protein is lost, free amino acids increase, and ammonia can accumulate (King et al., 1990). Major changes in gene expression in the tip tissues of the spears occur within 6 h of harvest, including de novo induction of specific genes (King and Davies, 1992).

Asn levels increase markedly in tips of spears within 24 h of harvest (King et al., 1990; Hurst et al., 1993). Synthesis of amide amino acids and other nitrogen-containing compounds during periods of stress has been noted many times (Sieciechowitz et al., 1988; Rabe, 1990). However, the functional significance of amide synthesis is unclear (Rabe, 1990). AS (EC 6.3.5.4), the primary enzyme responsible for Asn synthesis, has proved difficult to characterize biochemically, due to its instability in vitro (Lea and Miflin, 1980) and the presence in many plant extracts of both AS inhibitors (Joy et al., 1983) and asparaginase activity (Streeter, 1977). Indeed, alternative pathways for Asn synthesis have been proposed for asparagus, because no AS activity could be detected (Cooney et al., 1980).

We have previously constructed cDNA libraries from tips of asparagus spears at harvest and from tips of harvested spears held in the dark at 20°C for 12 h. Differential hybridization screening of these libraries isolated several cDNA clones that had altered expression following harvest, including four that increased substantially in prevalence by 12 h (King and Davies, 1992). We report here the identification and analysis of one of these cDNA clones that encodes a harvest-induced AS transcript.

MATERIALS AND METHODS

The growth conditions for plant material (*Asparagus officinalis* L. cv Limbras 10), spear harvest and storage conditions, and the methods for RNA isolation and analysis were as previously described (King and Davies, 1992). The term spear "tip" refers to the apical 30 mm of 180-mm spears. Fleshy roots were obtained from field-grown plants, washed with water to remove the soil, and frozen in liquid nitrogen within 10 min of excision. The roots were divided into two types: developing (white) and mature (brown). pTIP12 was used as the probe for RNA studies. It is a partial-length cDNA clone for AS, of identical nucleotide sequence to the corresponding region of pTIP27 (our unpublished data). The cDNA libraries were constructed and screened with DNA probes as described by King and Davies (1992).

Nucleotide sequence analysis was carried out by the chain termination method of Sanger et al. (1977), using either a Sequenase kit (United States Biochemical) or a commercial service (Lofstrand Laboratories Limited, Gaithersburg, MD). The sequence was substantiated by sequencing of both strands of the cDNA, and no ambiguities remain. Amino acid alignment of deduced sequences was carried out using the FASTA computer program (Pearson and Lipman, 1988).

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Abbreviation: AS, asparagine synthetase.

RESULTS

Isolation of a cDNA Clone Encoding AS

The cDNA pTIP12 is a partial clone encoding a harvest-induced transcript from the tips of asparagus spears. DNA nucleotide sequence analysis of pTIP12 revealed high homology of its derived amino acid sequence to that of the *Pisum sativum* cDNAs AS1 and AS2, which encode AS (Tsai and Coruzzi, 1990). The pTIP12 cDNA was used to screen 1 × 10^5 clones from an unamplified asparagus cDNA library, constructed from tips of harvested spears stored at 20°C for 12 h, and six independent homologous clones were obtained. The longest cDNA clone, pTIP27, was chosen and its entire nucleotide sequence was determined.

pTIP27 is a full-length cDNA clone encoding an mRNA for AS from asparagus spear tips. The encoded mRNA is 1978 bp long, with 55 bp untranslated 5′ and 153 bp untranslated 3′ sequence, including a poly(A) extension of 53 bp. Starting with the first in-frame Met, the derived protein is 590 amino acids long (Fig. 1), with a predicted molecular mass of 66.5 kD and an isoelectric point of 6.3. The asparagus sequence is 81% identical to AS1 from *Pisum*, 58% identical to AS from *Escherichia coli*, and 44% identical to the human AS (Fig. 1). Allowing for conservative amino acid substitutions, the similarities are 89%, 74%, and 64% for the *Pisum*, *E. coli*, and human sequences, respectively. The homology between the two plant sequences extends throughout, except for a divergence over the final 30 to 40 amino acids. The greatest region of divergence between the plant and the human and *E. coli* sequences is between residues 129 and 209 (135 and 240 in Fig. 1). The first four amino acids of the human and plant sequences (Met-Cys-Gly-Ile) form an amino acid motif that has been determined to be the Gln-binding region for the human enzyme (van Heeke and Schuster, 1989).

Expression of AS in Harvested Asparagus Spears

We have previously demonstrated that transcript levels for AS (using pTIP12, a partial clone for AS) are low in growing

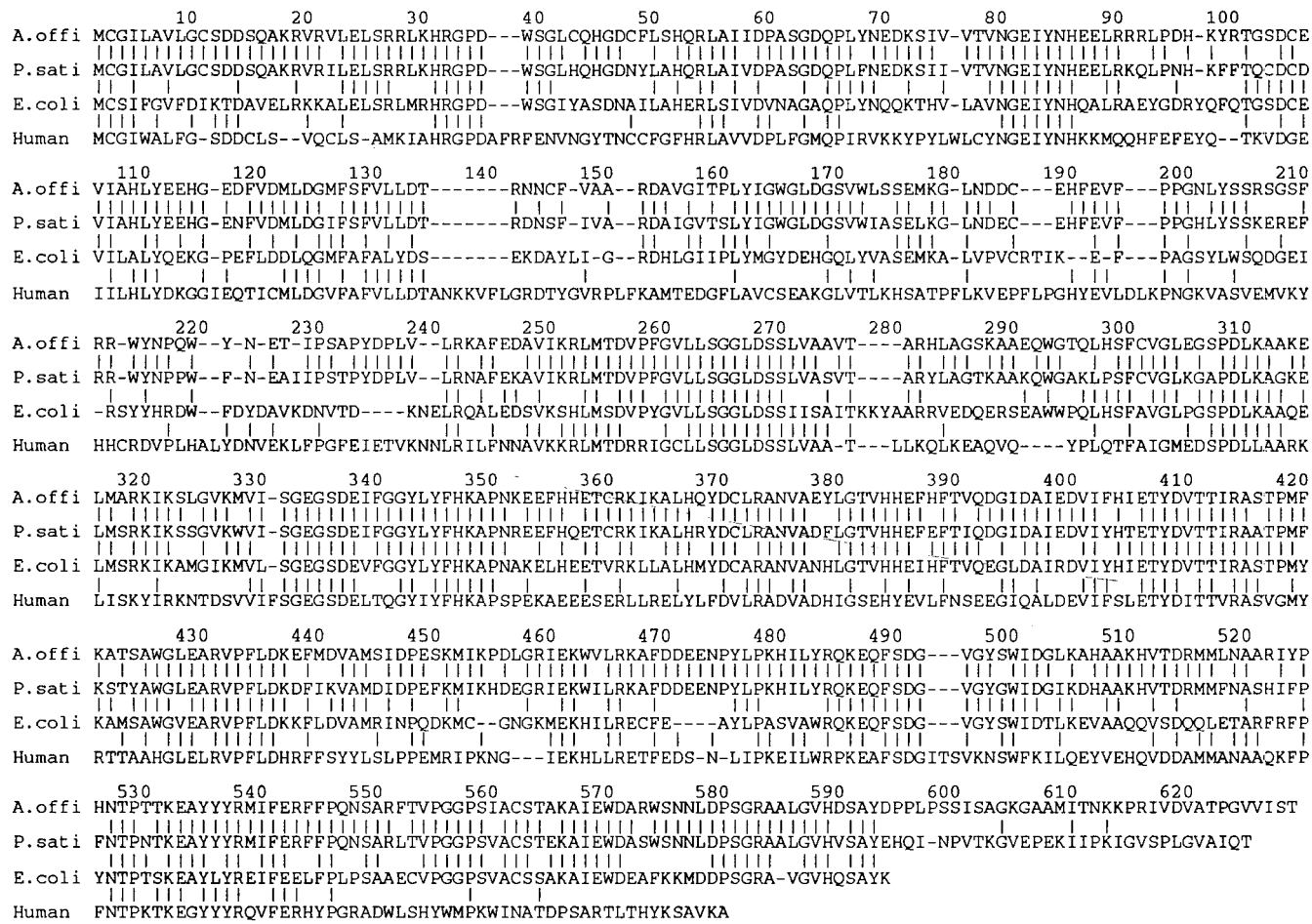

Figure 1. Amino acid sequence homology between a monocotyledonous plant (*A. officinalis*; pTIP27), a dicotyledonous plant (AS1 from *P. sativum*; Tsai and Coruzzi, 1990), *E. coli* (Scofield et al., 1990), and human (Andrulis et al., 1987) AS polypeptides. Bars denote identities between the *A. officinalis* and the *P. sativum*, *E. coli*, or human sequences. Dashes in the amino acid sequences represent gaps introduced to maximize alignment of the polypeptides. Numbering refers to total residues on each line of the figure rather than to a specific sequence.

spear tips but increase dramatically within 6 h of harvest (King and Davies, 1992). Because transcript levels were high at the first time point previously examined (6 h after harvest), the temporal and spatial abundance of AS transcripts was investigated in more detail. RNA was isolated at seven time points up to 12 h and analyzed by dot-blot hybridization. At each time point, the 180-mm spear was divided into three sections; tip (apex to 30 mm), mid (75–105 mm), and butt (150–180 mm). The tip contains actively dividing meristematic cells, grading into a zone of cell elongation in the mid region, and the butt comprises mature tissue in which the vascular material is lignified (Lill et al., 1990).

AS transcript abundance increased first in the tips, 2 h after harvest, and subsequently in the remainder of the spear at 4 h (Fig. 2).

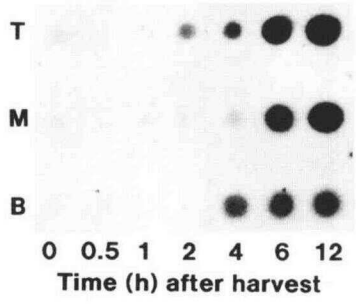

Figure 2. Temporal abundance of mRNAs for AS in harvested asparagus spears. Total RNA was prepared at each time from spears harvested and held in light at 20°C for up to 12 h. Total RNA (10 μg) was blotted onto Hybond-N$^+$ membranes (Amersham International Pty.) following the manufacturer's recommendations, and transcript abundance was detected by hybridization to [^{32}P]dCTP-radiolabeled pTIP12 insert and visualized by autoradiography. The spear was divided into three regions: tip (T), mid (M), and butt (B).

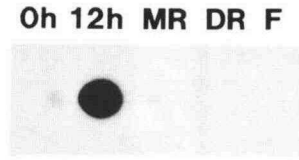

Figure 3. Abundance of mRNAs for AS in different tissues of asparagus. Total RNA (10 μg) was blotted onto Hybond-N$^+$ membranes (Amersham International Pty.) following the manufacturer's recommendations, and transcript abundance was detected by hybridization to [^{32}P]dCTP-radiolabeled pTIP12 insert and visualized by autoradiography. RNA was isolated from tips of spears at harvest (0 h), tips from spears harvested and held at 20°C for 12 h in the dark (12 h), green fern (F), mature fleshy roots (MR), and developing fleshy roots (DR).

Expression of AS in Different Tissue Types

To determine further the specificity of the AS response, and to try to locate tissues in which AS might have other functions, we measured the abundance of AS transcripts in a range of tissue types. RNA was prepared from green fern cladophylls (formed as the spear matures into a fully photosynthetic structure) and developing and mature fleshy roots, which were obtained from the crown during the period of spear production. The fleshy roots constitute the bulk of the underground portion of the asparagus plant and mobilize carbohydrate, water, and soil nutrients to support spear growth in the spring (Robb, 1984). Only very low levels of transcript for AS were detected in any of these tissues (Fig. 3).

DISCUSSION

Asn was the first amino acid to be discovered in plants, being isolated from *Asparagus sativus* in 1806 (Vauquelin and Robiquet, 1806). However, the mechanisms underlying synthesis of Asn have proved difficult to define (Cooney et al., 1980; Sieciechowitz et al., 1988). Although Asn can reach 40 mM in asparagus seedlings (Cooney et al., 1980), AS activity in this plant has not been demonstrated. We report here the isolation of a cDNA clone for AS from asparagus spear tips.

Only low levels of transcript for AS could be detected in growing spears, roots, or fern. However, AS mRNA levels began to increase in the tips of spears by 2 h after harvest and in the other sections of the spear after 4 h. This suggests that all sections of the harvested spear were responding to a particular postharvest signal. The induction of AS transcript in tips of harvested spears precedes the accumulation of Asn, which increases from 6 to 12 h onward (Hurst et al., 1993). Asn accumulates in many plants under stress conditions (Rabe, 1990) and may help prevent ammonia toxicity in these situations (Givan, 1979; Rabe, 1990). It has been suggested that AS uses ammonia directly (van Heeke and Schuster, 1989) and that high levels of ammonia can induce the expression of genes of the nitrogen cycle (Miao et al., 1991). Ammonia begins accumulating in tips of asparagus spears 24 to 48 h after harvest (King et al., 1990), well after induction of the AS gene (Fig. 2). This strongly suggests that induction of the AS gene is unrelated to changes in tissue ammonia levels.

AS genes in *Pisum* have been shown to be up-regulated during the dark period of the day/night cycle and to be dark-induced in other tissues, including roots (Tsai and Coruzzi, 1990, 1991). AS expression in asparagus spears is not greatly affected by the light environment either pre- or postharvest (King and Davies, 1992), and only very low levels of transcripts for AS were detected in fleshy roots, ferns, or spears before harvest (Fig. 3). The regulation of AS gene expression in asparagus spears is markedly different from that reported for *Pisum*, suggesting that the enzyme may be playing a role in harvested asparagus spears that is different than those in other plants studied so far.

Depletion of respiratory substrate imported from the crown is one of the very early stresses perceived by asparagus spears after harvest. Suc content declines dramatically in tips within 3 h of harvest (D.E. Irving, P.L. Hurst, unpublished data), paralleling respiratory decline (King et al., 1990). Genix et al. (1990) found that Suc starvation of sycamore cell cultures led to accumulation of Asn, a situation that is reversible by the addition of Suc, and that the level of Asn correlated with the amount of protein metabolized. We propose that the AS gene

is induced in harvested asparagus spears in response to carbohydrate stress. Asn accumulation in harvested spears may represent a strategy to conserve nitrogen in a translocatable, carbon-efficient manner, as occurs during dark-induced leaf senescence (Thomas, 1978).

The isolation of a cDNA clone for AS from asparagus provides a valuable tool for the study of Asn synthesis in this plant, the subject of over 180 years of research. The factors controlling the activity of the gene in asparagus may differ markedly from those described for AS in other systems.

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The EMBL/GenBank/DDBJ accession number for the sequence (pTIP27) reported in this article is X67958.

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