Seasonal Variation of Proteins and Amino Acids in Apple Flower Buds (*Malus pumila* Mill., cv. McIntosh/M7)[1]

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The contents of total protein, amino acids, and 4-hydroxyproline-rich glycoproteins of spur buds of apple trees (*Malus pumila* Mill. cv. McIntosh/M7) have been determined from bud inception to bud break by use of analytical chromatographic methods developed to quantitate the unique and other amino acids that occur in these proteins. All amino acids, except aspartic acid, changed in concentration during the season. When the amino acid data were grouped into classes with distinct properties, the seasonal fluctuations of hydrophilic amino acids and hydrophobic residues (ratio R3) ranged from 1.93 in late August to 2.28 in the following spring. Spur buds also vary considerably in their total protein content during the season and ranged from 4.4% to 10.3% on a dry weight basis. The 4-hydroxyproline-rich glycoprotein content of the primary cell walls of buds can also be determined from the amounts of 4-hydroxyproline found in their acid hydrolysates. The glycoproteins found in the extracellular matrices of apple flower buds ranged from a low of 3.81% of the total protein during the late spring (April) to a high of 4.84% of the total protein in the previous autumn, corresponding to 0.39% and 0.22%, respectively, on a dry weight basis.

The level of nitrogenous compounds in deciduous fruit trees changes substantially during autumnal leaf senescence when much of the leaf nitrogen is translocated into spurs and smaller twigs and thence into older shoot bark (Murneek and Logan, 1932; Oland, 1963; Spencer and Titus, 1972; O'Kennedy et al., 1975; Kang and Titus, 1980). Amino acids stored in the woody tissue of fruit trees are of particular importance in the early stages of spring growth since at this time environmental conditions for absorption and translocation of nutrients are not always optimal (Batjer and Rogers, 1952; Boynton, 1954). This reserve nitrogen is utilized early in the spring for the development of flowers and leaves (Tromp and Ovaa, 1971, 1973; O'Kennedy and Titus, 1979). Most studies on nitrogen metabolism in apple trees report changes of amino acids and soluble protein in shoots, bark, or leaves, but little is known of the variation in the levels of these cellular constituents in the buds themselves.

The molecular mechanisms that control higher plant growth and development, dormancy, and remarkable adaptation to low temperatures are not well understood (Goldberg, 1987; Tseng and Li, 1987). It is not known, for example, how meristems originate and give rise to the morphological pattern specific for vegetative organ systems (leaf, stem, root) and floral organ systems (petal, stamen, pistil), nor is it known how the underlying molecular processes can regulate differential gene expressions that determine flower patterns in higher plants (Kamalay and Goldberg, 1980; Singer and McDaniel, (1986). There is some evidence that oligosaccharide fragments from plants' cell walls may play a role in flowering (Tran Thanh Van et al., 1985); however, this result awaits confirmation.

The seasonal cycle of soluble cellular constituents in relation to cold tolerance in deciduous trees and other plant species, on the other hand, has been well documented (Levitt and Siminovitch, 1940; Siminovitch and Briggs, 1949; Siminovitch, 1963; Pomeroy et al., 1970; Steponkus, 1984; Guy et al., 1985; Tseng and Li, 1987; Yelenoski et al., 1987) and in general indicates an autumn increase in soluble proteins, sugars, and nucleic acids. While these studies have emphasized that the development of cold tolerance in shoots, bark, or leaves is closely associated with a general augmentation of total soluble proteins in the protoplasm, including augmentation of cell organelles and other membranous components of the cell (Pomeroy et al., 1970; Singh et al., 1977a, 1977b; Johnson-Flanagan et al., 1986; Johnson-Flanagan and Singh, 1987), there is little information on the insoluble cell wall or membranous proteins of higher plants, and very little has been done toward studying the problem from the biochemical point of view. The work of Lamport (1980) demonstrated that the extracellular matrices of the primary cell walls of angiosperms are comprised of glycoproteins in which a number of proline residues are posttranslationally modified to 4-*trans*-hydroxyproline. The 4-hydroxyproline-rich glycoproteins, i.e., extensins, arabinogalactan proteins, and salt-extractable glycoproteins and agglutinins, which are major structural components of the primary cell walls of all dicotyledonous plants (Lamport and Epstein, 1983; Fincher et al., 1983; McNeil et al., 1984; Cooper et al., 1987; Averyart-Fullard et al., 1988), are difficult to extract with conventional protein solvents. Jian et al. (1987) have recently found increased levels of glycoproteins at the cell surface of different hardy wheat varieties, suggesting that one of their functions is to increase freeze tolerance in plants.

The present study attempts to establish the levels and variation of total protein as well as the individual amino acids in apple spur buds (*Malus pumila* Mill. cv. McIntosh/M7) from bud inception (July) to the first pink stage (April). The aims were (1) to obtain information on quantitative fluctuations of these cellular constituents in this species, (2) to clarify the importance of each amino acid during flower induction and accumulation of storage proteins in overwintering apple buds, and (3) to ascertain whether or not similar seasonal changes occur in the total

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amount of 4-hydroxyproline-rich glycoproteins found in the extracellular matrices of the primary cell wall of apple flower buds.

MATERIALS AND METHODS

Materials. Type W-3 cation-exchange spherical resin, sized to 9.0 ± 0.5 μm, was obtained from Beckman Instruments Inc., Palo Alto, CA, while type DC-6A, 11.0 ± 1.0 μm spherical resin, was purchased from Dionex Corp., Sunnyvale, CA. L-Tryptophan, D-glycosamine hydrochloride, D-galactosamine hydrochloride, and 4-hydroxyproline were obtained from Calbiochem-Behring Corp., LaJolla, CA. 3-Nitro-L-tyrosine was purchased from Aldrich Chemical Co., Milwaukee, WI, and was purified as described previously (Zarkadas et al., 1987). The standard amino acid calibration mixture, Piercesolve (ethylene glycol monomethyl ether), ninhydrin, and stannous chloride hydrate were purchased from Pierce Chemical Co., Rockford, IL. Sodium citrate dihydrate (crystals) was obtained from Allied Fisher Scientific, Fairlawn, NJ. All other chemicals and reagents were of the highest purity commercially available and were used without further purification.

Experimental Procedure. *Collection of Plant Material and Sample Preparation.* Buds were collected from six 30-year-old apple trees, *M. pumila* Mill. cv. McIntosh/M7, at the Macdonald College of McGill University orchard, Ste. Anne de Bellevue, Quebec. Forty buds from spurs on 2- to 3-year-old wood, assumed to be flower buds (90%), from each tree were combined and quickly frozen at −170 °C. All tissues were lyophilized for 24 h, ground in a standard electrically driven Thomas-Wiley Intermediate Model mill equipped with a 64-mm stainless steel hopper and stationary blades (A. H. Thomas Co., Philadelphia, PA), and stored in small plastic bottles at −20 °C for subsequent analysis.

Extraction Procedure for Apple Flower Bud Tissues. To remove all traces of soluble amino acids and other compounds from apple flower bud tissues, samples (2–3 g) of the pulverized tissues were extracted with a mixture of 0.1 M HCl in 75% ethyl alcohol (Rangeley and Lowrie, 1976). The samples were suspended in 200 mL of extraction solvent and homogenized for 3 min in a VirTis Model 45 (VirTis, Gardiner, NY) homogenizer (speed set at 30/100), and the homogenates were centrifuged at 50000*g*(SS-34 Sorvall rotor) for 30 min at 2 °C. The supernatants were removed and dried under vacuum (Buchi, Rotavapor, Switzerland) at 45 °C. The pellet was suspended in the same extraction solvent, and the extraction procedure was repeated twice. The final pellets were suspended in 20 volumes of acetone, and the suspension was again centrifuged as before. The pellets from the final centrifugation were dried at 50 °C overnight and then placed under vacuum to remove the last remnants of solvent. The dried pellets were finally ground to pass through a 40-mm screen and stored at −20 °C until needed.

Procedures for Amino Acid Analyses. Amino acid analyses were carried out on a semiautomated amino acid analyzer (Beckman Spinco Model 120C) using single-column methodology as described previously (Zarkadas et al., 1986).

Seasonal changes in amino acids and proteins of apple flower buds were determined on 50-mg samples of lyophilized powders. Samples were hydrolyzed in Pyrex test tubes (18 × 150 mm) under vacuum (below 10 μmHg) with 15.0 mL of triple-glass-distilled constant-boiling HCl (6.0 M) at 110 °C in duplicate for each of four times, 24, 48, 72, and 96 h, respectively, with the usual precautions described by Hunt (1985) and Zarkadas et al. (1988).

The data reported for serine, threonine, and tyrosine represent the average of values extrapolated to zero time of hydrolysis. Addition of phenol (10–15 μL) to the hydrolysates usually prevented chlorination of tyrosine. The values for valine, isoleucine, leucine, and phenylalanine are averages of data from 48, 72, and 96 h of hydrolysis. All others are reported as the average values from 24, 48, 72, and 96 h of hydrolysis as described previously (Zarkadas et al., 1988).

The 4-hydroxyproline was determined separately from concentrated hydrolysate (equivalent to 0.1 mg of protein/analysis) by the procedure of Zarkadas et al. (1986). Recoveries of 4-hydroxyproline were calculated relative to alanine. Methionine and cyst(e)ine were determined separately (50 mg) by the performic acid procedure of Moore (1963). The recovery of cystine plus cysteine as cysteic acid and of methionine as the methionine *S*,*S*-dioxide was calculated relative to the yields obtained by the performic acid treatment of standard solutions of these amino acids and relative to alanine and leucine present in the samples.

Tryptophan in apple flow bud samples (50 mg) was also determined separately after alkaline hydrolysis (Hugli and Moore, 1972) by a rapid method (Zarkadas et al., 1986), using 3-nitrotyrosine as the internal standard.

Data processing and regression analysis of the amino acid data were carried out by the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS, 1985).

Predicting Properties of Proteins from Amino Acid Compositions. Since amino acid compositions represent a large body of easily accessible data not clearly related in any simple way to useful structural properties of proteins, it would be useful if there were unequivocal ways of grouping the amino acids into classes with distinct properties in the hope that such classes correlate to some extent with the rather general properties of the proteins in mixtures. One feature of protein structures that is fairly reliable is the tendency of the side chain of charged or very polar amino acid residues to be external, interact strongly with water, and have high solubility in water. At the opposite end of the polarity scale are the apolar or hydrophobic side chains, which tend to have low solubility in water and therefore will be internal (Bigelow, 1967; Nozaki and Tanford, 1971).

One feature of protein structures that should be attainable from amino acid composition is predicting whether a protein or protein mixture is associated with membranes, and thus should have a larger number of nonpolar residues and fewer charged residues, or whether a protein mixture is located in the cytosol in which case it should resemble a typical soluble nonmembrane protein(s). Barrantes (1973, 1975) has grouped the amino acids into four classes, total charged, hydrophilic, hydrophobic, and apolar, and simply compared the ratio (R) of the frequencies of occurrence (χ) of whatever particular side chains of proteins one wishes to stress, e.g.

$$R = \sum_k \chi_k / \sum_j \chi_j \qquad (1)$$

where k can be hydrophilic and j hydrophobic side chains or k polar and j nonpolar as defined by Barrantes (1973): basic, histidine + lysine + arginine; acidic, aspartic + glutamic + asparagine + glutamine; total charged, basic + acidic; hydrophilic, total charged + threonine + serine; hydrophobic, valine + methionine + isoleucine + leucine + tyrosine + phenylalanine + tryptophan; apolar, hydrophobic − tyrosine; ratio 1 (R1), hydrophilic/hydrophobic; ratio 2 (R2), hydrophilic/apolar; ratio 3 (R3), total charged/hydrophobic; and ratio 4 (R4), total charged/apolar.

Although the particular choice of residues used to construct these ratios is somewhat arbitrary (Barrantes, 1973, 1975) one particular ratio scale that reliably weights the tendency of charged or very polar residues to be external is R3. This ratio (R3) is convenient because it spreads out different proteins over a wide scale range, from 0.36 to 2.03, and gives a measure with more information about the system.

Determination of 4-Hydroxyproline Glycoproteins. Total protein in each apple flower bud acid hydrolysate was determined by the procedure of Horstmann (1979) as described previously (Nguyen et al., 1986, Zarkadas et al., 1988). In this study, an attempt was also made to relate the amounts of protein-bound 4-hydroxyproline, which occurs exclusively in the 4-hydroxyproline-rich glycoproteins of the primary cell walls of the angiosperms, i.e., extensin, arabinogalactan protein, and salt-extractable glycoproteins (Lamport, 1980; McNeil et al., 1984; Cooper et al., 1987), to the contents of these extracellular matrix proteins in apple flower buds.

Previous studies (Zarkadas et al., 1988) have shown that a general method to calculate the amount of a specific protein j present in apple flower buds from the quantitative determination of a given unique amino acid i known to occur exclusively in that specific protein (j) is

$$P_j = C_i \frac{[1000]}{n'_i} \frac{\mathrm{WE}(P_j)}{M_r(i)} \qquad (2)$$

where P_j is the concentration of a specific primary cell wall glycoprotein j, i.e., extensin, expressed in grams per kilogram of total

protein, C_i is the mean concentration of a unique protein-bound amino acid, i, i.e., 4-hydroxyproline, in grams per kilogram of total protein, WE(P_j), is the weight equivalent of a specific protein j determined from its known amino acid composition according to Horstmann (1979), and n'_i is the number of residues of a unique amino acid residue i per 1000 amino acid residues.

Since the 86-kDa carrot extensin monomer has been the best characterized (Cooper et al., 1987), its amino acid composition reported by Stuart and Varner (1980) and Van Holst and Varner (1984) has been used as a standard for quantitating the 4-hydroxyproline-rich glycoprotein content in apple flower buds in this study. This quantitation is based on three major findings: first, that the 86-kDa carrot extensin monomer (ext-1) consists of 35% protein and 65% carbohydrate; second, that the 30-kDa protein moiety contains 306 amino acids in its primary sequence (Chen and Varner, 1985a,b; Smith et al., 1986) and has a calculated mean residue weight of WE = 0.1095 ng/nmol (Horstmann, 1979); third, that 4-hydroxyproline makes up 45.5% of the polypeptide backbone, corresponding to 455 4-hydroxyproline residues/1000 amino acid residues. The anhydrous $M_r(i)$ of 4-hydroxyproline is 113.12.

Substituting the computed parameters for extensin in eq 2, the total 4-hydroxyproline-rich glycoproteins in grams per kilogram of total protein in apple flower buds can be calculated by the following analytical convention:

$$\text{amt extensin } (P_{\text{ext-1}}) = \text{amt Pro(4-OH)} \times 2.128 \qquad (2a)$$

RESULTS AND DISCUSSION

Flower buds of 30-year-old apple trees (cv. McIntosh/M7) were chosen as an example of a potentially hardy tree tissue because it would be expected that the amplitude of protein change in the cells of the vegetative shoot meristem will be greatest and easiest to detect in this fast-growing tissue, even in autumn or winter, when tree buds are exposed to the greatest extremes of low temperatures, +10 to −50 °C or lower. Accurate determination of total protein and amino acids in apple spur buds, at various stages of growth and development, was carried out at the nanomole range by use of analytical chromatographic methods described previously (Zarkadas et al., 1986), and the results obtained are summarized in Table I. The analytical capability of the present methodology and its application to complex tissue hydrolysates, i.e., apple flower buds, have been presented previously (Khanizadeh et al., 1987, 1988).

Seasonal Cycle of Amino Acids. The seasonal changes of amino acids in the buds and levels of statistical significance obtained from analysis of variance as presented in Table I represent the average values of six replicates and duplicate determinations obtained from duplicate 24-, 48-, 72-, and 96-h acid hydrolysates. These values show deviations of less than ±3.0% from the average values obtained between replicates within the same sampling period. The least variability occurred when the amino acid data were expressed as grams of amino acids per 100 g of anhydrous fat- and ash-free protein. The main advantage of this unit of expressing the composition of a complex protein mixture is that comparison can be made on a protein basis and that the percentage recovery by weight can be found by simple summation (Tristram and Smith, 1963; Zarkadas et al., 1988). The mean protein concentration of each sample was determined by the procedure described by Horstmann (1979). The mean residue weight (WE, μg/nmol) and conversion factors F and F' (μg/nmol) obtained are listed in Table I and can be used in all subsequent quantitations of the same tissue following standard procedures as described by Horstmann (1979), Peterson (1983), and Zarkadas et al. (1988).

The results show that although the amino acid profiles of apple flower buds at various stages of development appeared to be similar and highly characteristic of this plant tissue, there was a preferential accumulation of in-

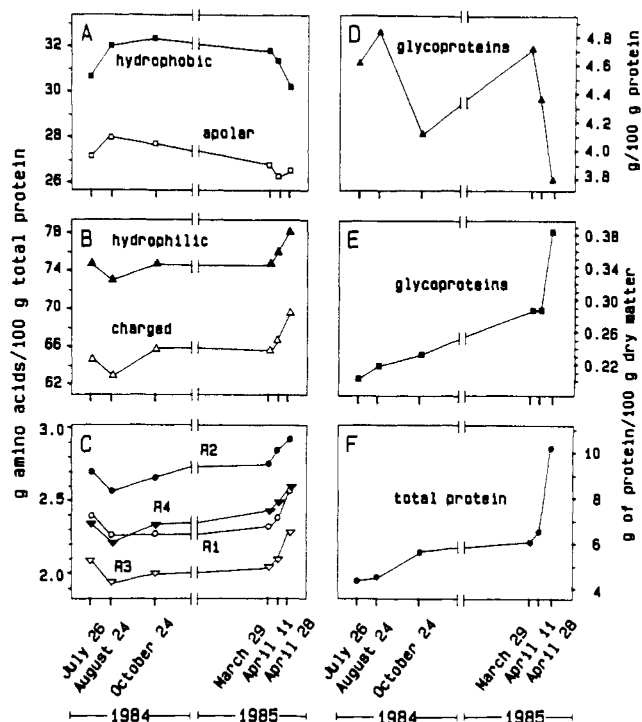

Figure 1. Seasonal variation of proteins and amino acids in apple flower buds (*M. pumila* Mill. cv. McIntosh/M7): A, hydrophobic (■—■) and apolar (□—□) amino acids; B, hydrophilic (▲—▲) and total charged (△—△) amino acids; C, ratios defined by Barrantes (1973) as R1, hydrophilic/hydrophobic (○—○), R2, hydrophilic/apolar (●—●), R3, total charged/hydrophobic (▽—▽), R4, total charged/apolar (▼—▼); D, 4-hydroxyproline-rich glycoproteins expressed on a total protein basis (▲—▲); E, 4-hydroxyproline-rich glycoproteins expressed on a dry weight basis (■—■); F, total protein (●—●).

dividual amino acids in buds throughout the season (Table I). The seasonal variation of amino acids in apple bud tissues was found to be highly significant ($P < 0.01$) for each amino acid analyzed, except for aspartic acid. The variation in amino acids per unit tissue protein, which occurs principally in the period from August 24 to October 24 and again from March 29 to April 11, is in the absence of cell division in accord with the assumption that these changes reflect changes per cell or unit number of cells.

It would therefore be useful if the constituent amino acids of apple flower buds could be grouped into classes with distinct properties so that such classes correlate to some extent with the general properties of the proteins in this plant tissue. As mentioned earlier, Barrantes (1973, 1975) has grouped the amino acids into four classes, totally charged, hydrophylic, hydrophobic, and apolar, and simply compared the ratio (R) of the frequencies of occurrence of whatever particular side chains of proteins one wishes to stress, especially in terms of the proportion of total charged and hydrophobic residues (ratio R3). This method of amino acid classification was used, and the results obtained are summarized in Table I; the salient features are illustrated graphically in the composite Figure 1.

The figure shows the normal fluctuation of the totally charged, hydrophylic, hydrophobic, and apolar amino acids and compares the ratios (R1–R4) of the frequencies of their occurrence characterizing the seasonal processes of the buds. From these data, it is apparent that the total charged or hydrophilic amino acid content of apple flower buds decreased abruptly in late summer and began a gradual increase again in the autumn, which was probably maintained throughout winter and the early spring of the following year. It appears therefore that the seasonal

Table I. Seasonal Changes of Protein-Bound Amino Acids and Proteins in the Flower Buds of Apple Trees, *M. pumila* (Mill. cv. McIntosh/M7) (Grams of Amino Acid/100 g of Total Protein)

	July 26, 1984		August 24, 1984		October 24, 1984		March 29, 1985		April 11, 1985		April 28, 1985		signif level betw test periods	
amino acid	mean ± SEM[a]	CV	mean ± SEM[a]	CV	mean ± SEM[a]	CV	mean ± SEM[a]	CV	mean ± SEM[a]	CV	mean ± SEM[a]	CV	CV	F^a
aspartic acid	12.45 ± 0.27	6.17	12.60 ± 0.26	5.89	12.05 ± 0.24	5.68	12.04 ± 0.36	10.30	11.90 ± 0.21	6.00	12.37 ± 0.17	3.81	6.89	0.99^{ns}
threonine	4.68 ± 0.10^{c-e}	5.86	5.13 ± 0.09^{b}	4.20	4.36 ± 0.07^{e}	4.63	4.59 ± 0.13^{d,e}	9.60	4.88 ± 0.09^{b-d}	6.60	5.03 ± 0.27^{b,c}	15.00	8.59	4.20**
serine	5.01 ± 0.08^{b}	4.22	4.91 ± 0.12^{b}	7.11	4.47 ± 0.08^{c}	5.11	4.42 ± 0.12^{c}	9.00	4.36 ± 0.07^{c}	5.81	4.00 ± 0.16^{d}	11.11	7.26	10.72**
glutamic acid	11.12 ± 0.14^{d}	3.64	10.24 ± 0.11^{e}	2.76	12.19 ± 0.04^{c}	0.98	12.12 ± 0.13^{c}	3.72	12.24 ± 0.21^{c}	6.01	13.20 ± 0.19^{b}	4.01	4.10	36.12**
proline	4.73 ± 0.09^{b}	5.54	4.67 ± 0.06^{c}	2.86	4.70 ± 0.10^{b}	5.87	4.68 ± 0.04^{b,c}	2.82	4.74 ± 0.05^{c}	3.81	3.97 ± 0.06^{c}	4.15	4.22	19.69**
glycine	5.16 ± 0.08^{b}	4.32	4.77 ± 0.17^{c}	9.42	5.23 ± 0.12^{b}	6.56	4.99 ± 0.08^{b,c}	5.44	4.72 ± 0.12^{c}	8.81	4.74 ± 0.09^{c}	5.10	6.86	3.94**
alanine	5.45 ± 0.07^{b}	3.38	5.39 ± 0.09^{b}	3.97	4.67 ± 0.05^{d}	3.11	4.98 ± 0.03^{c}	2.03	4.62 ± 0.05^{d}	4.08	5.44 ± 0.20^{b}	10.33	5.17	21.77**
cysteine	1.15 ± 0.02^{b,c}	5.11	1.21 ± 0.11^{b}	22.42	1.05 ± 0.03^{c}	8.94	1.12 ± 0.02^{b,c}	6.32	1.23 ± 0.02^{b}	5.82	1.19 ± 0.01^{b}	2.41	10.33	2.81*
valine	5.92 ± 0.05^{c}	2.50	5.92 ± 0.12^{c}	5.13	5.50 ± 0.06^{d}	3.19	5.61 ± 0.07^{d}	4.25	6.16 ± 0.04^{b}	2.28	6.27 ± 0.10^{b}	4.72	3.77	16.77**
methionine	2.88 ± 0.10^{c}	9.63	3.16 ± 0.11^{b}	8.82	2.87 ± 0.04^{c}	4.26	2.73 ± 0.04^{b,c}	5.45	2.64 ± 0.07^{d}	9.32	2.94 ± 0.03^{c}	2.95	7.29	7.19**
isoleucine	5.31 ± 0.08^{b}	4.12	5.04 ± 0.06^{c}	3.46	5.44 ± 0.05^{b}	2.79	5.10 ± 0.04^{c}	2.90	4.81 ± 0.06^{d}	4.28	4.63 ± 0.07^{e}	4.38	3.66	23.04**
leucine	6.92 ± 0.13^{d}	5.31	8.17 ± 0.08^{b}	2.42	8.07 ± 0.19^{b,c}	6.81	7.88 ± 0.09^{b,c}	3.84	7.78 ± 0.10^{c}	4.65	7.80 ± 0.10^{c}	3.57	4.57	12.47**
tyrosine	3.50 ± 0.04^{d}	3.63	4.06 ± 0.38^{c}	25.65	4.65 ± 0.06^{b}	3.46	5.03 ± 0.08^{b}	5.16	5.08 ± 0.04^{b}	2.71	3.63 ± 0.33^{c,d}	25.40	12.28	15.46**
phenylalanine	5.51 ± 0.06^{b}	3.17	5.01 ± 0.16^{c}	8.19	5.34 ± 0.10^{b}	5.01	4.96 ± 0.08^{c}	5.78	4.43 ± 0.09^{d}	6.76	4.67 ± 0.05^{d}	3.05	5.67	19.21**
histidine	2.79 ± 0.05^{b}	5.54	2.70 ± 0.06^{b}	6.34	2.65 ± 0.05^{b,c}	4.83	2.50 ± 0.06^{c}	7.85	2.50 ± 0.05^{c}	6.83	2.81 ± 0.09^{b}	8.60	6.57	5.70**
lysine	6.39 ± 0.18^{d}	8.12	6.40 ± 0.23^{d}	9.62	7.17 ± 0.15^{c}	5.97	7.45 ± 0.09^{c}	4.32	7.43 ± 0.07^{c}	3.34	8.08 ± 0.23^{b}	7.89	6.36	17.26**
arginine	7.65 ± 0.20^{b}	7.46	7.17 ± 0.14^{c}	5.63	6.66 ± 0.16^{d}	6.92	6.59 ± 0.06^{d}	3.22	7.48 ± 0.06^{b,c}	2.93	6.79 ± 0.10^{d}	4.28	5.11	14.65**
tryptophan	0.66 ± 0.04^{b}	18.05	0.70 ± 0.02^{b}	8.70	0.52 ± 0.01^{c}	2.75	0.53 ± 0.01^{c}	7.15	0.50 ± 0.01^{c}	8.82	0.29 ± 0.01^{d}	13.21	11.16	47.53**
hydroxyproline	2.17 ± 0.13^{b}	16.37	2.27 ± 0.07^{b}	7.39	1.94 ± 0.04^{b,d}	6.29	2.22 ± 0.06^{b}	8.74	2.06 ± 0.07^{b,c}	11.88	1.79 ± 0.08^{d}	12.52	19.96	5.55**
ammonia	3.52 ± 0.08^{b}	6.16	2.76 ± 0.20^{c}	20.80	2.80 ± 0.08^{c}	7.61	2.40 ± 0.11^{d}	16.08	2.21 ± 0.10^{d}	16.08	3.18 ± 0.14^{b}	12.22	13.69	16.04**
total AA N	19.10 ± 0.08^{e}	1.23	18.30 ± 0.18^{e}	2.58	18.18 ± 0.04^{d}	0.61	17.83 ± 0.11^{c,d}	2.13	17.86 ± 0.06^{c}	1.20	18.70 ± 0.15^{b}	2.23	1.79	21.20**
WE, μg/nmol	0.1087 ± 0.001^{e}	0.31	0.1091 ± 0.001^{d,e}	0.79	0.1096 ± 0.001^{c}	0.16	0.1097 ± 0.001^{b,c}	0.15	0.1102 ± 0.001^{b}	0.58	0.109 ± 0.001^{c,d}	0.59	0.48	8.99**
F	0.1105 ± 0.001^{d}	0.36	0.1109 ± 0.001^{c,d}	1.01	0.1112 ± 0.001^{c}	0.22	0.1113 ± 0.001^{c}	0.13	0.1120 ± 0.001^{b}	0.59	0.1110 ± 0.001^{c,d}	0.62	0.55	6.56**
F'	0.1199 ± 0.001^{d}	0.42	0.1205 ± 0.001^{c,d}	0.89	0.1204 ± 0.001^{c,d}	0.61	0.1209 ± 0.001^{b,c}	0.29	0.1215 ± 0.001^{b}	0.41	0.1189 ± 0.001^{e}	0.77	0.57	16.14**
basic	16.83 ± 0.37^{c}	2.24	16.27 ± 0.86^{c}	5.31	16.49 ± 0.29^{c}	1.76	16.54 ± 0.58^{c}	3.52	17.42 ± 0.33^{b}	1.80	17.68 ± 0.89^{b}	5.04	3.48	8.26**
acidic	47.13 ± 1.01^{c,d}	2.15	45.67 ± 1.36^{d}	2.99	48.50 ± 1.37^{c}	2.83	48.32 ± 1.87^{c}	3.88	48.28 ± 2.78^{c}	5.77	51.14 ± 1.77^{b}	3.45	3.93	7.26**
charged	63.96 ± 0.89^{c}	1.41	61.94 ± 1.22^{d}	1.96	64.98 ± 1.49^{c}	2.31	64.86 ± 1.49^{c}	2.30	65.70 ± 2.60^{c}	3.96	68.82 ± 1.59^{c}	2.32	2.65	13.88**
hydrophobic	30.70 ± 0.53^{d}	1.73	32.06 ± 0.82^{b,c}	2.56	32.40 ± 0.94^{b}	2.92	31.83 ± 0.85^{c,d}	2.69	31.39 ± 0.42^{c}	1.34	30.24 ± 0.67^{d}	2.32	2.30	10.80**
hydrophilic	73.64 ± 0.86^{c,d}	1.17	71.98 ± 1.31^{d}	1.82	73.82 ± 1.60^{c}	2.17	73.87 ± 2.08^{c}	2.83	74.95 ± 2.39^{c}	3.19	77.85 ± 1.15^{b}	1.48	2.37	10.08**
apolar	27.20 ± 0.49^{c,d}	1.83	28.00 ± 0.57^{d}	2.05	27.75 ± 0.80^{c}	2.89	26.80 ± 0.83^{d,e}	3.11	26.31 ± 0.36^{d}	1.37	26.21 ± 0.69^{d,e}	2.63	2.40	9.66**
R1	2.40 ± 0.05^{c}	2.30	2.25 ± 0.09^{d}	4.20	2.28 ± 0.11^{d}	4.98	2.32 ± 0.12^{d}	5.42	2.39 ± 0.09^{c}	3.75	2.58 ± 0.08^{b}	3.20	4.16	11.56**
R2	2.71 ± 0.06^{d}	2.32	2.57 ± 0.07^{e}	2.77	2.66 ± 0.13^{d,e}	4.95	2.76 ± 0.16^{c,d}	5.83	2.85 ± 0.11^{b,c}	3.83	2.93 ± 0.09^{b}	3.24	4.18	10.58**
R3	2.08 ± 0.05^{c}	2.49	1.93 ± 0.08^{d}	4.17	2.01 ± 0.10^{c,d}	5.05	2.04 ± 0.09^{c}	4.74	2.09 ± 0.09^{c}	4.35	2.28 ± 0.09^{b}	4.19	4.29	13.78**
R4	2.35 ± 0.05	2.50	2.21 ± 0.06	2.35	2.35 ± 0.11	5.03	2.42 ± 0.12	5.14	2.50 ± 0.11	4.46	2.55 ± 0.07	2.80	4.11	14.66**
total protein, g/100 g DWB	4.43 ± 0.75^{e}	4.82	4.61 ± 0.07^{e}	5.95	5.71 ± 0.56^{d}	2.75	6.16 ± 0.52^{c,d}	2.93	6.62 ± 0.82^{c}	4.31	10.29 ± 1.79^{b}	13.14	8.70	121.22**
extracell matrix glycoprotein (eq 2a):														
g/100 g total protein	4.622 ± 0.268^{b}	16.37	4.839 ± 0.13^{a}	7.39	4.129 ± 0.092^{b,c}	6.29	4.728 ± 0.119^{b}	8.74	4.37 ± 0.15^{b,c}	11.88	3.807 ± 0.169^{d}	12.52	10.96	5.55**
g/100 g DWB	0.203 ± 0.009^{e}	13.15	0.222 ± 0.024^{d,e}	3.10	0.236 ± 0.007^{d}	8.37	0.291 ± 0.005^{c}	6.59	0.289 ± 0.008^{c}	10.31	0.388 ± 0.123^{b}	9.21	9.05	59.17**

[a] Mean values and standard error of the means (SEM) for 3 replicates and 24 determinations. Significance: F, values from analysis of variance; **, $P < 0.01$; *, $P < 0.05$. Key: ns, not significant; CV, coefficient of variation. [b–e] Means along a horizontal column with different superscripts are significantly different at the $P < 0.01$ level by Duncan's new multiple-range test (Duncan, 1955). [f] Calculated according to Heidelbaugh et al. (1975). [g] The total protein, WE, F, and F' constants were calculated according to Horstmann (1979), where F is the apparent average residue molecular weight increased in proportion to the missing tryptophan and cyst(e)ine values, while F' was also calculated for determining protein mass in the absence of tryptophan, cyst(e)ine, proline, and 4-hydroxyproline. DWB, dry weight basis. [h] Calculated according to Barrantes using eq 1. [i] Data for 4-hydroxyproline-rich glycoproteins were calculated from the amounts of 4-hydroxyproline found in the acid hydrolysates of apple flower buds according to eq 2a and represent the mean values for 12 determinations from 3 replicates.

fluctuations of these two distinct classes of amino acids have a reciprocal relationship. While the observed changes in the levels of these two classes of amino acids from July 26 to August 24 may be correlated with flower bud induction in apple trees, the abrupt increase in the levels of hydrophobic amino acids and the small but sharp decline in the hydrophilic amino acid content of apple flower buds found to occur from August 24 to October 24 (Figure 1A,B) may be part of the seasonal process of acclimation of the tree to low temperatures.

During the dormant period, between 24 October and 24 March, there was little change in the amino acid content of buds (Figure 1). In early spring there was a slight increase in total charged and hydrophilic amino acids followed by a rapid increase in April just before the flower buds burst (Figure 1B); concurrently, the overall hydrophobic amino acid content of apple flower buds declined to a minimum in April. Reserve amino acids and nitrogen are of great importance in apple buds at bud break, a time when no new photosynthetic material is available for the synthesis of new carbon compounds. Although it is not possible at this point to assess the significance of these transitory seasonal variations in either individual or groups of amino acids, or to clearly understand the molecular mechanism regulating the series of biochemical reactions leading to seasonal fluctuations in the amino acid profiles of apple flower buds, the small but significant augmentation in the contents of hydrophobic amino acids and total protein of buds in the autumn and early spring may be part of seasonal processes of adaptation of the apple tree to low temperatures. The major increase in protein content in late spring (Figure 1), just before bud break, is probably due to the translocation of proteins or amino acids from the tree bark into the growing points of the flower buds (Kang and Titus, 1980; Titus and Kang, 1982).

Seasonal Cycle of Proteins. The results presented in Table I show that the protein content of buds started to increase just before cold acclimation and after the fruit had been harvested. Unlike bark storage nitrogen, which increases before tree dormancy and decreases in early spring (Siminovitch, 1963; Titus and Kang, 1982), bud storage proteins increased constantly throughout the season. As may be seen in the composite Figure 1F, the increases in protein content (grams/100 g dry weight basis) occurred principally from August 24 to October 24 and continued during the winter into early spring (March 29), while the major change in net protein synthesis occurs in the period from March 29 to April 28. It is not known what triggers these synthetic processes, but the net effect is a gradual increase in protein content within the existing cells, which may change their physical properties so that it enables them to withstand or adapt to adverse low temperatures of −20 to −50 °C or lower, prevalent in the north temperate zone. The results are in accord with those reported by other investigators (Kacperska-Palasz et al., 1977; Brown and Bixby, 1975; Trunova and Zvevera, 1977; Chen and Li, 1980).

To ascertain whether or not other soluble proteins and protoplasmic proteins of the apple flower buds are increased during dormancy and adaptation to low temperatures, as suggested by Siminovitch (1963) and Pomeroy et al. (1970), the ratio R3 was calculated (Figure 1C). The results of Table I show that from July 26 to August 24 there is a change in the R3 ratio and hydrophobicity of the proteins in the apple flower buds. This suggests that some of the newly synthesized proteins in August must have higher levels of hydrophobic amino acids in their primary sequence but lower solubility and fewer charged amino acids. It should also be noted that the R3 ratios of the same protein mixtures in October and March are markedly lower in comparison with the high R3 values obtained in late April (Figure 1C), which coincides with the period of increased protein synthesis in the apple flower buds (Figure 1F) at that time. Although the identity and biological significance of these newly synthesized proteins during dormancy has not yet been established, nor is it known how the underlying molecular processes can regulate differential gene expressions that determine protein transformation patterns in the buds (Singer and McDaniel, 1986), further detailed studies to ascertain their nature, function, and location in the plant cells, including cell organelles and other membranous protein components of the cell, may prove a very fruitful area for future research.

The presence of considerable amounts of the unique amino acid 4-hydroxyproline in the acid hydrolysates of apple flower buds, ranging from 1.79 to 2.27 g/100 g of total protein throughout the season (Table I), is highly significant. The values obtained for protein-bound 4-hydroxyproline per unit of protein show low coefficients of variation between replicates, and within the precision of the present methodology (±3.0%), recoveries were found to be quantitative (Table I), indicating that the analytical errors were also small relative to the biological variability ($P < 0.01$) observed between bud tissue harvested at different intervals during the season. From the known distribution of 4-hydroxyproline in the primary sequence of the 30-kDa glycoprotein moiety of carrot extensin (Stuart and Varner, 1980; Van Holst and Varner, 1984; Chen and Varner, 1985a,b), which has been used as a standard for comparison in the present study (Varner and Lin, 1989), the content of 4-hydroxyproline-rich glycoproteins of apple flower buds was calculated by multiplying the amounts of 4-hydroxyproline found in their acid hydrolysates by 2.128 (eq 2a), as described previously (Khanizadeh et al., 1988).

The data on protein composition, as presented in Table I on a dry weight basis (DWB), show that the 4-hydroxyproline-rich glycoprotein content of apple flower buds vary significantly ($P < 0.01$) throughout the season, ranging from a low of 0.21% (DWB) during the summer to a high of 0.39% (DWB) in the following spring. As may be seen in composite Figure 1E, the increase in 4-hydroxyproline-rich glycoproteins occurs principally in the period from July 26 to August 24, after which it gradually increased, which was maintained throughout the winter, while the major change in glycoprotein content appears to occur in the period from April 11 to April 28. From a comparison of the seasonal cycles of glycoproteins and the total protein in apple flower buds (Figure 1E,F), it appeared that a relatively close relationship existed between the seasonal variation in 4-hydroxyproline-rich glycoprotein content and the seasonal cycle of total proteins, when the results were expressed on the basis of unit dry weight of tissue. However, when the 4-hydroxyproline-rich glycoprotein content of buds was expressed on a total protein basis, it was evident that the seasonal increase in primary cell wall glycoproteins is not a gradual one but consists of two peaks (Figure 1D), one which coinciding with the time of increase in hydrophobic amino acids in the buds (Figure 1A) and the other a smaller peak slightly preceding the period of most intense synthesis of hydrophilic amino acids and protein from April 11 to April 28 (Figure 1B,F). The results obtained on the seasonal cycle of proteins in apple buds are compatible with those reported by Siminovitch (1963) on black locust bark and Li et al. (1965) on dogwood. These authors have consistently demonstrated, for example, that a close correlation exists

between seasonal variation in protein content and winter hardiness in these two deciduous tree species. Similar studies on apple trees have not been reported previously. Nevertheless, the striking and consistent biochemical changes observed in the flower buds of apple trees during the early autumn and spring test periods, pointing to an increase in many macromolecular components, suggest that augmentation of total protein, including the 4-hydroxyproline-rich glycoproteins of the primary cell wall, must in some way increase the adaptation of the bud cells to withstand the stress of the winter temperatures of the north temperate zone. The biochemical mechanism by which this increase in freezing tolerance is achieved remains to be elucidated.

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Registry No. Aspartic acid, 56-84-8; threonine, 72-19-5; serine, 56-45-1; glutamic acid, 56-86-0; proline, 147-85-3; glycine, 56-40-6; alanine, 56-41-7; cysteine, 52-90-4; valine, 72-18-4; methionine, 63-68-3; isoleucine, 73-32-5; leucine, 61-90-5; tyrosine, 60-18-4; phenylalanine, 63-91-2; histidine, 71-00-1; lysine, 56-87-1; arginine, 74-79-3; tryptophan, 73-22-3; hydroxyproline, 51-35-4.

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