Expression, Cloning, and Immunological Analysis of Buckwheat (*Fagopyrum esculentum* Moench) Seed Storage Proteins†

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cDNA of buckwheat (*Fagopyrum esculentum* Moench) was isolated from immature seeds harvested 14 days after pollination. Two genes, designated *FA02* and *FA18*, were found to encode legumin-like proteins and were expressed during seed development. The deduced amino acid sequence of FA02 was identical to the N-terminal amino acid domain of BW24KD, which was believed to be a major buckwheat allergen (Urisu, A.; Kondo, Y.; Morita, Y.; Yagi, E.; Tsuruta, M.; Yasaki, T.; Yamada, K.; Kuzuya, H.; Suzuki, M.; Titani, K.; Kurosawa, K. Isolation and characterization of a major allergen in buckwheat seeds. In *Current Advances in Buckwheat Research*; Shinshu University Press: Matsumoto, Japan, 1995; pp 965−974). It was predicted that FA02 would be cleaved to generate two separate components, a 41.3 kDa α-subunit and a 21 kDa *β*-subunit. Antiserum was raised against the deduced FA02 *β*-subunit, and immunoblotting of total protein from buckwheat seeds (*F. esculentum* M. and *Fagopyrum tartaricum* Gaertn.) revealed that several groups of proteins reacted with the antiserum. Polypeptides in the 23−25 kDa range displayed the greatest reactivity.

Keywords: *Fagopyrum esculentum M.; Fagopyrum tartaricum G.; buckwheat; legumin; allergen*

INTRODUCTION

Buckwheat (*Fagopyrum esculentum* Moench) is known as a valuable source of protein, and chemical analyses of hydrolysates indicate that the amino acid composition of buckwheat grain is nutritionally superior to that of cereal grains (*1*). However, reports of serious anaphylactic reactions following ingestion of buckwheat have been reported in Japan (*2*) and other countries (*3*, *4*). Some proteins found in buckwheat seed have been reported to be major allergens (*5*, *6*), but they have not yet been characterized at the molecular level.

In this study we describe the characterization of buckwheat genes expressed during seed development. One of the genes, *FA02*, had a high degree of homology with other legumin-like proteins and an amino acid sequence identical to the N-terminal amino acid domain of a major buckwheat allergen. The region of the deduced *β*-subunit of FA02 was subcloned into an expression vector. Antiserum was raised against the expressed polypeptide, and buckwheat proteins that reacted with the antiserum were isolated and characterized.

MATERIALS AND METHODS

Plant Materials. Buckwheat grains (*F. esculentum* M. cv. Kitayuki and Kitawase and *Fagopyrum tartaricum* Gaertn.) were obtained from Hokkaido National Agricultural Experiment Station. For the construction of a cDNA library and Northern blot analysis, immature cv. Kitayuki seeds grown in a greenhouse (16 h of light, 20−25 °C) were harvested 7, 14, 21, and 28 days after manual pollination (DAP). Hypocotyls and cotyledons were obtained 7 days after sowing from seedlings grown in the dark at 25 °C.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from developing seeds, flowers, buds, hypocotyls, and cotyledons according to the method of Stiekema et al. (*7*). Three micrograms of each RNA sample was separated on a formaldehyde gel, blotted onto charged nylon filters (Pharmacia BioTech, Uppsala, Sweden), and hybridized with cDNA probes using standard protocols (*8*). Northern analysis images were visualized with a Bio Imaging Analyzer (BAS 2000; Fuji Photo Film, Tokyo, Japan).

Construction of a cDNA Library. A cDNA library was constructed from poly A^+ RNA extracted from immature seeds collected 14 DAP. Poly A^+ RNA was isolated from total RNA using the oligotex-dT30 (Super) (Roche, Mannheim, Germany) batch method according to the manufacturer's directions. cDNA was prepared from 5 *μ*g of poly A^+ RNA using a directional cloning toolbox kit (Pharmacia BioTech) and a *Not*I−oligo dT primer. Finally, *Eco*RI/*Not*I-digested cDNA was ligated into Lambda-ExCell (Pharmacia BioTech), packaged using an in vitro packaging kit (Stratagene, LaJolla, CA), and plated with *Escherichia coli* strain NP66 (Pharmacia BioTech).

Cloning and DNA Sequencing Analysis. cDNAs corresponding to genes abundantly expressed in immature seeds were isolated from the 14 DAP cDNA library using labeled single-stranded cDNA probes synthesized from mRNAs isolated from 14 DAP immature seeds and germinated cotyledons. Plaques that showed strong hybridization signals with the 14 DAP cDNA probe, but not with the probe from germinated cotyledons, were selected, and the plasmid was rescued by in vivo excision (Pharmacia BioTech). These clones were tested by Northern blot analysis to determine whether they represent differential expression patterns in immature seeds and germinated cotyledons. An Auto Read sequencing kit and an ALF Express automatic DNA sequencer (both from Pharmacia BioTech) were used to determine the nucleotide sequences of the inserts.

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† The nucleotide sequence data reported will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under the accession numbers D87980 (*FA02*) and D87982 (*FA18*).

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10.1021/jf0011485 CCC: $20.00
Published on Web 03/15/2001

Subcloning and Expression of the *FA02* β-Subunit. The nucleotide region corresponding to amino acids 378−565 of *FA02* (β-FA02) was PCR-amplified using the primers 5′-GGA TCC GGA TTG GAG CAA GCG TTC TGC and 5′-CTG CAG TTA TTAGTG ATG GTG ATG GTG ATG CCC GAA ACG CTC CCT CTC CTT CTC ATC and cloned into pMal-cRI (New England BioLabs) downstream of and in frame with the maltose binding protein cassette. The resulting maltose binding protein−β-FA02 fusion protein was expressed in *E. coli* strain XL1-Blue (Stratagene) and purified from cytoplasmic proteins according to the manufacturer's directions. Purified protein was concentrated using the Millicap system (Millipore, Bedford, MA), and the protein concentration was determined by a Bradford assay (Bio-Rad, Herucles, CA) using bovine serum albumin as a standard. Production of antiserum against the predicted β-FA02 protein was performed by injecting 1 mg of the protein dissolved in 10 mM sodium phosphate (pH 7.6) into a 1-month-old female rabbit three times at 13-day intervals.

Polyacrylamide Gel Electrophoresis and Immunoblotting. Buckwheat seeds were ground to a powder in liquid N_2 with a mortar and pestle. The ground seeds were extracted with 200 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.4. Extracted samples were incubated at 90 °C for 5 min in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS) with and without 5% 2-mercaptoethanol to give reducing or nonreducing conditions, respectively. SDS-PAGE was carried out according to the method of Laemmli (*9*). The proteins were electroblotted onto a PVDF membrane (BioRad) using a semidry electroblotting apparatus (BioRad), at 2.5 mA cm^{-2} for 1.5 h (*10*). Blocking was done with 0.5% casein in phosphate-buffered saline, pH 7.4 (PBS), containing 0.2% Tween 20. The primary antibody was diluted 1:5000 in PBS and 0.5% casein, and the secondary antibody (AP-goat, anti-rabbit IgG, Zymed, San Francisco, CA) was diluted 1:2000. Detection was done with luminol and CDP-Star (Roche). The membranes were exposed to X-ray film (Fuji Photo Film) for 5−10 min.

RESULTS

A cDNA library was constructed using poly A^+ RNA from buckwheat seeds harvested 14 DAP. This library was screened with radioactively labeled single-stranded cDNA probes synthesized from mRNAs isolated from 14 DAP immature seeds and germinated cotyledons. Thirteen plaques screened from a total of 20000 showed strong hybridization signals with the 14 DAP cDNA probe but not with the probe from germinated cotyledons. Seven of these corresponded to a single gene family, as determined by comparisons of the nucleotide sequences from their 5′ termini, and were found to encode legumin-like proteins. Two of these genes, *FA02* and *FA18*, were selected as representative of the group for further analysis. Both DNA strands of these clones were sequenced; the *FA02* insert was1905 bp long, and *FA18* was 1691 bp in length. The largest open reading frame in *FA02* encoded a 565 aa polypeptide, whereas *FA18* contained a 504 aa ORF (Figure 1 a). The two deduced amino acid sequences were compared with the amino acid sequences of other legumin-like proteins in the SwissProt database. FA02 and FA18 shared 75% identity. FA18 exhibited the highest level of identity (41%) with pumpkin 11S globulin followed by rice glutelin type I (40%), garden pea legumin J (38%), sunflower helinathinin (38%), rape crucifelin (35%), and soybean glycinin 1 (33%) (Figure 1a).

The regions of these proteins that had the least similarity to FA18 were the VR1 regions; the VR1 motif was first observed and named by Simon et al. (*11*). FA02 contained tandem repeats of R(S/T)RQSESEE(S/F)-SRGDQ in this region (Figure 1a, amino acid positions 143−217). The other proteins contained a 20 residue N-terminal signal peptide and a consensus pattern for cleavage of the protein into two subunits (*12*). A single cysteine covalently links the α- and β-subunits in legumin-like proteins (*13*). This is also likely to be the case for FA02 and FA18, because the cysteine residue is strictly conserved (Figure 1a, FA02 amino acid positions 123 and 443). Only two other cysteine residues in the FA02 and FA18 subunits are as strictly conserved (Figure 1a, positions 47 and 80 in FA02). The conservation of these other cysteines suggested that they could be involved in an intrachain disulfide linkage (*13*). Accordingly, FA02 would be cleaved to give an acidic α-subunit of 41.3 kDa (p*I* 5.1) and a 21.1 kDa basic β-subunit (p*I* 9.6). Cleavage of FA18 would yield a 33.5 kDa acidic α-subunit (p*I* 5.1) and a 21.4 kDa basic β-subunit (p*I* 10.1).

Figure 1b shows the 29 N-terminal residues of the FA02 and FA18 β-subunits and the N-terminal amino acid sequence of BW24KD, which is known to be the major allergen in buckwheat (*5*). FA02 shows particularly high homology with the N-terminal BW24KD amino acid sequence with 26 of 27 residues being identical.

Figure 2 shows Northern blots done with buckwheat RNA isolated from tissues, including seeds at different stages of development; the blots were probed with two segments of *FA02*. The α-subunit probe contained bases 1−361, and the β-subunit probe comprised bases 1279−1905. The transcripts that hybridized the probes were ∼1.9 kb long. During the first 7 DAP, *FA02* transcripts were not detected. Subsequently, levels increased with the level of seed development (14 and 21 DAP) and then gradually decreased with seed maturation (28 DAP). *FA02* transcripts could not be detected in any other tissues (Figure 2).

Figure 3 shows the SDS-PAGE pattern of proteins from buckwheat seeds. Under nonreducing conditions, the majority of storage proteins were 49−63 kDa polypeptides (Figure 3a, lanes 1−3). Immunoblotting detected the FA02 β-subunit and identified it as the major storage protein (Figure 3a, lanes 4−6). Reduction of the storage proteins by 2-mercaptoethanol resulted in two separate polypeptide groups: a larger group of 32−43 kDa and a smaller group of 23−25 kDa (Figure 3b, lanes 1−3). Immunoblotting resulted in several bands being detected (Figure 3b, lanes 4−6), and the most abundant bands were 23−25 kDa in size. With the antiserum against the FA02 β-subunit, 35, 42−45, and 63 kDa peptides from *F. esculentum* were also detected, as was an apparently abundant 35-kDa polypeptide from *F. tartaricum*.

DISCUSSION

We have isolated two cDNA clones that show greatly enhanced expression in immature buckwheat seeds. *FA02* and *FA18* were found by homology searches to be legumin-like genes. In general, legumin-like proteins are known to be part of the 11−12S globulin stored in seed (reviewed in ref *14*). In dicotyledonous plants, the most common type of globulin is the 11−12S globulin, which is an oligomeric protein composed of two polypeptides, a 30−40 kDa α-subunit and 20 kDa β-subunit, linked by a disulfide bond. These subunits are processed from a 50−60 kDa precursor (*12*). In buckwheat, the salt-soluble globulin accounts for almost half of total seed proteins and is represented mainly by the 13S 280

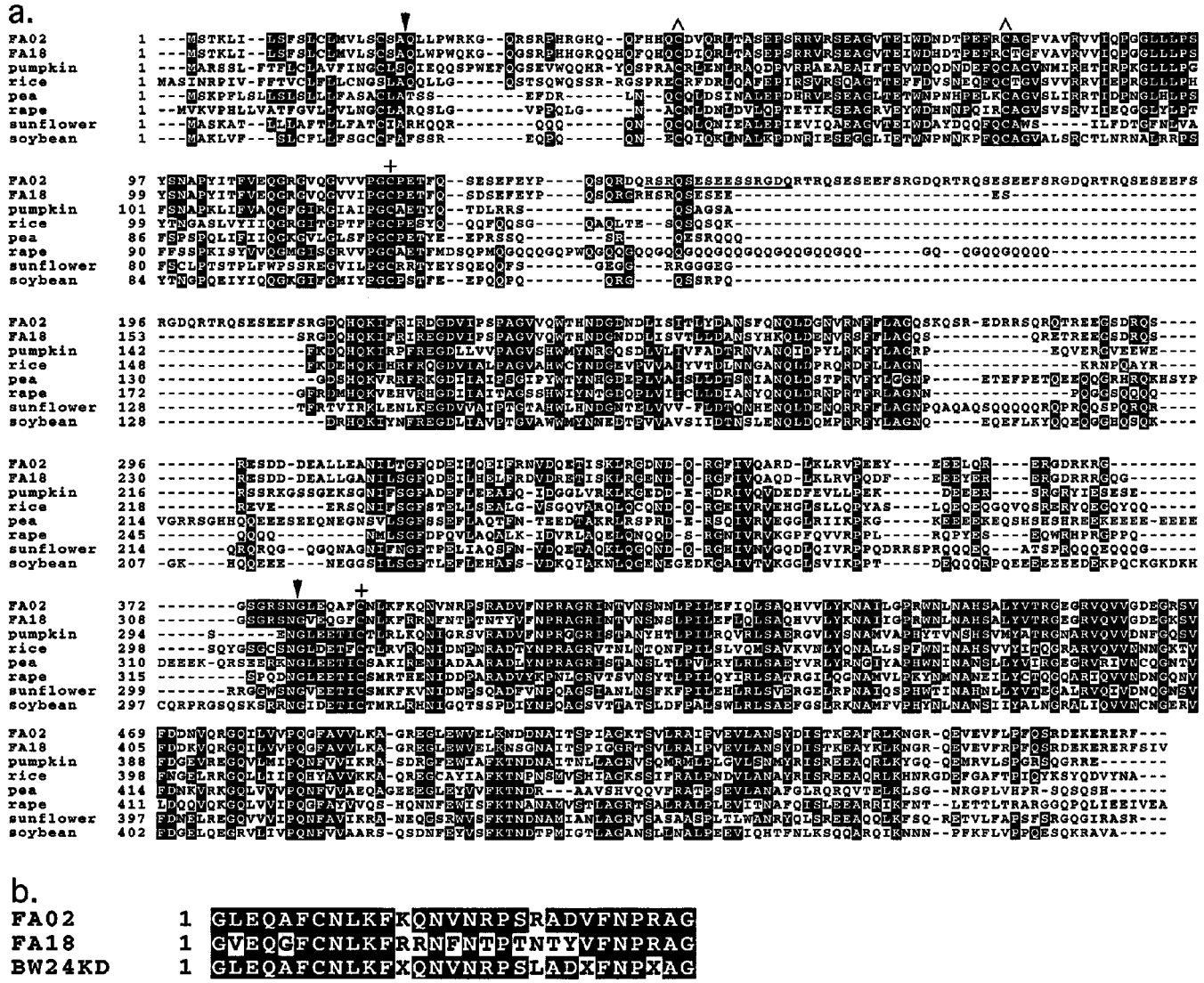

Figure 1. Alignment of amino acid sequences deduced from the cDNA sequences of *FA02*, *FA18*, and legumin-like genes. The sequences were aligned using BoxShade (http://www.ch.embnet.org/software/BOX_form.html); identical and similar residues are shown in reverse type and shaded boxes, respectively. (a) Sequences are FA02 (*F. esculentum*, accession number D87980), FA18 (*F. esculentum*, accession number D87982), *Cucurbita pepo* (pumpkin 11S globulin, accession number M36407), rice (*Oryza sativa* glutelintype I, accession number X05662), pea (*Pisum sativum* legumin J, accession number P05692), sunflower (*Helianthus annus* helianthinin, accession number P19084), rape (*Brassica napus* cruciferin, accession number J05233), and soybean (*Glycine max* glycinin G4, accession number S11004). Arrowheads indicate the predicted post-translational cleavage sites. Cysteine residues marked by a plus (+) are involved in interchain linkages, and those marked by a cap (^) may be involved in intrachain linkages. (b) Alignment is shown of some predicted amino acid sequences over their regions similar to FA02 residues 378−406, FA18 residues 314−342, and the N-terminal acidic domain of BW24KD. X = any amino acid.

kDa protein fraction (*15*). The total protein is composed of three size classes of polypeptides with molecular masses between 55 and 60 (α), 32−44 (*β*), and 16−29 kDa (*γ*) (*16*). The 57−58 kDa polypeptides (α) that can aggregate to form the major class of polypeptide in the 13S protein (known as 13S globulin) were classified as vicilin-like proteins (*17*). Furthermore, these polypeptides showed no reaction with antiserum against the FA02 *β*-subunit (Figure 3). The 32−43 and 23−25 kDa polypeptides, which are linked by a disulfide bond and constitute 13S globulin, were focused in the acidic and basic regions of an isoelectric focusing gel, respectively (*17*).

On the basis of the results of the analysis of in vitro and in vivo labeled translation products, Maksimovic et al. (*18*) suggested that the 13S globulin components were not the products of post-translational processing but were directly encoded by individual mRNAs. Northern analyses of *FA02* expression using the α- and *β*-subunit probes resulted in the appearance of the same-sized transcripts regardless of whether total or mRNA was used. The deduced amino acid sequences of FA02 and FA18 consisted of signal peptides as well as α- and *β*-subunits and showed conservation of the arrangement of the cysteine residues in comparison with legumin-like genes (Figure 1). Therefore, the components of buckwheat 13S globulin would be the products of post-translational processing, as is the case with legumin-like proteins. However, *F. esculentum* polypeptides of 35, 42−45, and 63 kDa reacted with antiserum against the FA02 *β*-subunit, and a *F. tartaricum* 35 kDa polypeptide was highly reactive to the antiserum (Figure 3). These polypeptides presumably included the common FA02 *β*-subunit epitope. In rice seeds, an unprocessed pro-glutelin reacts with antiserum against both the α- and *β*-subunits (*19*). The

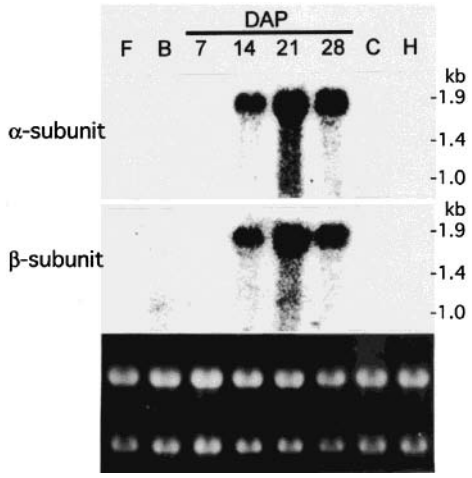

Figure 2. RNA blot analysis of *FA02* expression during seed development. Total RNA was extracted from buds (B), flowers (F), and immature seeds harvested on the indicated numbers of days after pollination (DAP). Seedling cotyledons (C) and hypocotyls (H) were harvested on the seventh day after germination. Three micrograms of total RNA was loaded in each lane. Numbers in the right-hand column indicate molecular weight markers (Promega). The filter was hybridized with probes corresponding to the α- and *β*-subunit regions of *FA02* (bases 1−361 and 1279−1905, respectively). Lowest panel is the ethidium bromide-stained formaldehyde gel.

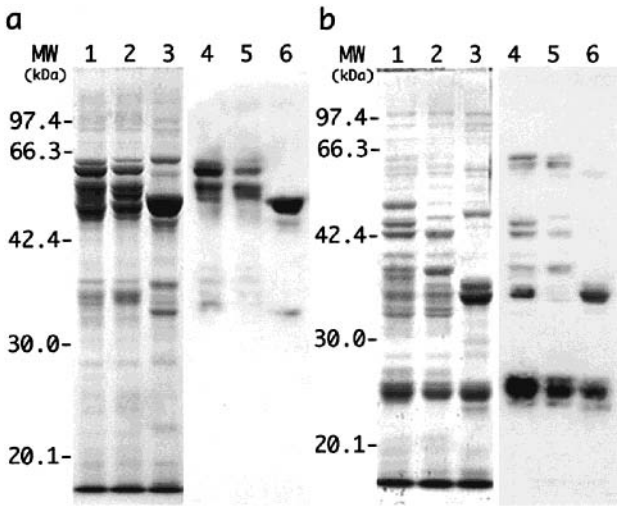

Figure 3. SDS-PAGE (lanes 1−3) and immunoblotting (lanes 4−6) of total soluble proteins from *F. esculentum* cv. Kitayuki (lanes 1 and 4) and cv. Kitawase (lanes 2 and 5) and *F. tartaricum* (lanes 3 and 6) under either nonreducing (a) or reducing conditions (b). Ten micrograms of protein was loaded in each lane. Molecular weights are indicated on the left (MW).

glutelin cDNA, which lacks the Asn-Gly processing site that is highly conserved among legumin-like proteins, has been cloned (*20*). In buckwheat, there is no evidence to indicate whether the 35, 42−45, and 63 kDa polypeptides are unprocessed forms of pro-polypeptides or FA02 homologues with different or without processing sites. In *F. esculentum*, the 42−45 and 63 kDa polypeptides were less reactive to the antiserum than the 23−25 kDa ones were; this may be the result of incomplete reduction.

The deduced FA02 amino acid sequence was found to have significant homology to BW24KD (Figure 1b), which was reported to be the major allergen that could bind IgE in sera from patients allergic to buckwheat (*5*). Furthermore, in an *E. coli* expression system, the translation product of a cDNA encoding the FA02 *β*-subunit was strongly recognized by sera from allergic patients (*21*). Uris et al. (*5*) reported that proteins 59−67, 34−37, and 26 kDa in size bound IgE in the sera of >30% of individuals allergic to buckwheat. Nair and Adachi (*22*) reported that 22, 36, 39−40, and 70−72 kDa proteins showed maximum IgE binding activity. These polypeptides were shown to have molecular masses similar to those that reacted with prepared anti-FA02 *β*-subunit antibodies (Figure 3b) and would have motifs similar to the FA02 *β*-subunit. It is important that the molecular characteristics of buckwheat allergens be determined so that ELISA performed with antiserum against the FA02 *β*-subunit may be used as a tool to breed nonallergenic buckwheat.

ABBREVIATIONS USED

DAP, days after pollination; ELISA, enzyme-linked immunosorbent assay; PVDF, poly(vinylidene difluoride); PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; bp, base pair; ORF, open reading frame; kb, kilobase pairs.

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Received for review September 19, 2000. Revised manuscript received January 10, 2001. Accepted January 22, 2001.

JF0011485