Plant Physiol. (1996) 112: 1301–1313

Oxidative Stress Results in Increased Sinks for Metabolic Energy during Aging and Sprouting of Potato Seed-Tubers[1]

G. N. Mohan Kumar and N. Richard Knowles*

Department of Agricultural, Food and Nutritional Science, 4–10 Agriculture/Forestry Center, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

Glutathione-mediated free-radical-scavenging and plasma membrane ATPase activities increase as sinks for metabolic energy with advancing tuber age. Plasma membrane ATPase activity from 19-month-old tubers was 77% higher than that from 7-month-old tubers throughout sprouting. The higher activity was not attended by an increase in the amount of ATPase per unit plasma membrane protein. Concentrations of oxidized (GSSG) and reduced glutathione more than doubled as tuber age advanced from 6 to 30 months, but the proportion of GSSG to total glutathione remained constant with age. The activity of glutathione transferase, an enzyme that catabolizes lipid-hydroperoxides, increased by 44 and 205% on a fresh weight and protein basis, respectively, as tubers aged from 6 to 30 months. Glutathione reductase activity also increased with advancing age, by 90% on a fresh weight basis and 305% on a protein basis. Older tubers had more glutathione reductase per unit of soluble and mitochondrial protein. The age-induced increase in cytosolic glutathione transferase activity was likely due to increased availability of lipid-hydroperoxides and/or a positive effector. Synthesis of glutathione requires ATP, and the increased reduction of GSSG resulting from catalysis of lipid-hydroperoxides is NADPH-dependent. Thus, increased plasma membrane ATPase and glutathione-mediated free-radical-scavenging activities likely constitute substantial sinks for ATP in older tubers prior to and during sprouting. Increased oxidative stress and loss in membrane integrity are central features of aging that undoubtedly contribute to the enhanced respiration of sprouting older tubers.

Potato (*Solanum tuberosum* L.) seed-tubers provide a model system for studies of the metabolic processes associated with aging of plant tissues in general and vegetative propagules in particular. Russet Burbank seed-tubers maintain their viability for about 3 years in cold storage (4°C, 95% RH); however, vigor and growth potential of tuber meristems depend on age. As tubers advance in age beyond about 7 months, apical dominance and plant vigor gradually decline, resulting in the production of many shoots per tuber, each with low specific leaf area and sparse root systems (Mikitzel and Knowles, 1990; Kumar and Knowles, 1993a). Paradoxically, older tubers have substantially higher rates of fully coupled, Cyt-mediated respiration than younger tubers at similar stages of sprout development (Kumar and Knowles, 1996). This greater ATP production by sprouting older tubers is required to maintain an AEC equal to that of sprouting younger tubers. Older tubers thus consume more ATP during sprouting to fuel metabolic processes that do not contribute directly to sprout growth (Kumar and Knowles, 1996). The nature of some of these age-induced and energetically expensive metabolic processes is the focus of this investigation.

Increased lipid peroxidation (Kumar and Knowles, 1993b) and progressive loss of membrane integrity are characteristics of aging tubers. As tuber age advances from about 6 to 24 months, membrane permeability to electrolytes increases, and this age-induced loss of membrane integrity is highly correlated with a decrease in the double-bond index of fatty acids within the membrane lipid fraction (Knowles and Knowles, 1989). Increased saturation of membrane lipids is known to cause organizational changes that disrupt membrane integrity and increase permeability (Barber and Thompson, 1980; Pauls and Thompson, 1981). Such changes in the membrane lipid microenvironment can also affect membrane protein mobility (Shinitzky and Inbar, 1976), potentially altering the activities and kinetic properties of membrane-bound enzymes, most notably transport proteins (Carruthers and Melchior, 1986). PM-bound ATPase is an enzyme with an activity subject to changes in its lipid environment (Cooke and Burden, 1990). Hence, a potential sink for ATP, which may be enhanced in sprouting older tubers, could be the ATPase in PMs that have become leaky with advanced age. In view of the increased energy-metabolizing ability of sprouting older tubers, we isolated PM vesicles and determined the effects of tuber age and sprouting on kinetic parameters of the membrane-bound ATPase.

Since increased free-radical-mediated peroxidation of fatty acids occurs during tuber aging (Kumar and Knowles, 1993b), glutathione-mediated free radical scavenging was also examined as a potential sink for metabolic energy (ATP and/or reducing equivalents) in sprouting older tubers. GSH is a potent inhibitor of lipid peroxidation and an efficient free radical scavenger (Haenen, 1989). In mammalian cells GSH can be as high as 2 to 10 mM and is directly responsible for maintaining the highly reduced intercellular environment and redox potential (Vasanthy, 1986). In

[1] This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to N.R.K.

* Corresponding author; e-mail rknowles@gpu.srv.ualberta.ca; fax 1–403–492–4265.

Abbreviations: AEC, adenylate energy charge; APase, acid phosphatase; GRase, glutathione reductase; GS, glucan synthetase; GSSG, oxidized glutathione; GTase, glutathione transferase; LDH, lactate dehydrogenase; LPC, lysophosphatidylcholine; PK, pyruvate kinase; PM, plasma membrane; POX, peroxidase; SOD, superoxide dismutase; UP, upper phase.

plants glutathione synthesis increases in response to various stress situations such as drought (Dhindsa and Matowe, 1981), chilling (Walker and McKersie, 1993), or injury by ozone (Lee and Bennet, 1982; Guri, 1983) or H_2O_2 (Smith et al., 1984; Smith, 1985). GSH is so important in the defense against activated oxygen species (Foyer and Halliwell, 1976) that its depletion is known to sensitize tumor cells to oxidative cytolysis (Arrick et al., 1982). As a POX, GTase is involved in protecting cells from the damaging effects induced by lipid-hydroperoxide accumulation and in the detoxification of cyto- and genotoxic compounds (Mauch and Dudler, 1993). Thus, the ability of cells to cope with oxidative stress depends in part on the de novo synthesis of GSH and/or its regeneration from GSSG. Synthesis of GSH is ATP-dependent and its regeneration from GSSG is catalyzed by GRase, with the consumption of NADPH (Carlberg and Mannervik, 1985). Tanaka et al. (1994) suggested that GRase is responsible for the reduction of more than 90% of cellular GSSG. Furthermore, enhanced consumption of NADPH during imbibition of aged seeds may limit the availability of reducing equivalents for metabolic processes that lead directly to germination (De Vos et al., 1994). We therefore compared the extent of NADPH consumption by the glutathione-mediated free-radical-scavenging system in young and old tubers during sprouting by quantifying glutathione (GSH/GSSG) levels and associated enzyme activities. PM ATPase and increased oxidative stress were identified as two sinks for metabolic energy that are enhanced in response to tuber aging.

MATERIALS AND METHODS

Certified potato (*Solanum tuberosum* L. cv Russet Burbank) seed-tubers, obtained directly from a local grower at the time of harvest, were stored up to 30 months under conditions inhibitory to sprouting (4°C, 95% RH). For discussion, 6- to 7-month-old tubers are referred to as physiologically "young" and 18- to 30-month-old tubers are referred as physiologically "old" or "aged." Tuber age was calculated from the time of harvest. Characterization of growth potential and physiological status of the young and old tubers during sprouting was accomplished by comparing sprout dry matter and respiration rates, respectively.

Whole-Tuber Respiration

The respiration of 7- and 19-month-old tubers was followed over a 10-d sprouting interval, as described previously (Kumar and Knowles, 1993c), with minor modifications. Treatments consisted of two tuber ages and three harvest dates (0, 5, and 10 d of sprouting), each replicated three times. Uniform-sized (180–200 g) 7- and 19-month-old tubers were selected from 4°C storage, rinsed, weighed, and sealed in 1.5-L high-density polyethylene chambers equipped with inlet and outlet ports (three tubers per chamber). Humidified air flow to each chamber was 125 mL min^{-1}. Respiration rates were determined by measuring CO_2 released from the tubers with a gas chromatograph (model 5890A, Hewlett-Packard) on a daily basis for 10 d. Since trends in respiration rates over the 10-d interval were similar to those previously reported (Kumar and Knowles, 1996), only the rates at 0, 5, and 10 d of sprouting are given, because these were the times selected for isolation of PMs and comparison of ATPase activities between the young and old tubers. Sprout number and dry weight at 10 d were recorded. A second respiration study utilizing 6-, 18-, and 30-month-old tubers was also conducted. Tubers from this study were selected at 0, 5, and 10 d of sprouting for comparison of glutathione metabolism.

Isolation and Characterization of PMs

PM vesicles were isolated from tuber tissue by modifying the methods of Iswari and Palta (1989). After the appropriate sprouting interval, tubers were removed from the respiration chambers and the sprouts were removed. An equal portion of the outer cortical tissue from the apical halves of each of the three tubers was excised (extending from the periderm inward 1 cm) and collectively grated into 50 mL of cold (4°C) grinding medium in the reservoir of a food processor (100 g of total tissue per treatment). The grinding medium consisted of 75 mM Mops buffer (pH 7.8) containing 250 mM Suc, 5 mM EGTA, 2 mM PMSF, 2 mM salicylhydroxamic acid, 2.5 mM $Na_2S_2O_5$, 1.5% (w/v) PVP, 0.5% (w/v) protease-free BSA, 10 μg mL^{-1} butylated hydroxytoluene, and 1 mM DTT (all chemicals from Sigma). The tissue was then extracted with a mortar and pestle in an additional 50 mL of grinding medium and filtered through two layers of Miracloth (Calbiochem), and the filtrate was centrifuged at 20,000*g* for 25 min. The resulting supernatant was centrifuged at 105,000*g* for 45 min to pellet microsomal membranes. The microsomal pellet was suspended in 9 mL of 10 mM potassium phosphate buffer (pH 7.8) containing 250 mM Suc. Three milliliters of the microsomal suspension was washed in 20 mL of phase-dilution buffer (5 mM Mops, pH 7.8, containing 250 mM Suc, 1 mM EGTA, 0.5 mM PMSF, 10 μg mL^{-1} butylated hydroxytoluene, 1 mM DTT, and 10 mM KCl), and the microsomes were pelleted at 105,000*g* for 45 min. The microsomal pellet was suspended in 5 mL of phase-dilution buffer and stored at −80°C. PMs were isolated from the remaining 6 mL of the microsomal suspension by a two-step, PEG-DEX (5.8%, w/w) two-phase system containing 15 mM NaCl. The microsomes (6 mL) were added to 24 g of the two-phase system at 4°C, the mixture was inverted 40 times, and phase separation was facilitated by centrifugation (2,500*g* for 20 min) in a swinging-bucket centrifuge (GLC-1, Sorvall). Upper and lower phases were diluted 4 and 10 times (v/v), respectively, with phase-dilution buffer and centrifuged at 105,000*g* to pellet the membrane vesicles. All manipulations were at 4°C. The UP pellets were suspended in 5 mL of phase-dilution buffer and stored at −80°C.

Differential sensitivity of the ATPase to azide, molybdate, nitrate, and vanadate was used to further characterize microsomal and UP membrane vesicles. ATPase activity was determined by colorimetric measurement of Pi released from ATP hydrolysis (Serrano et al., 1976). The reaction medium consisted of 5 mM ATP (vanadium-free,

Sigma), 5 mM PEP (to regenerate ATP), 5 mM $MgCl_2$, and, where indicated, 5 mM NaN_3 (an inhibitor of mitochondrial ATPase), 100 mM KNO_3 (an inhibitor of tonoplast ATPase), 1 mM $NaMoO_4$ (an inhibitor of APases), 100 μM Na_3VO_4, 0.1 mg mL^{-1} LPC, and 24 units of PK (Sigma) in 10 mM Pipes buffer (pH 6.5). The membrane suspension (10 μL of microsomes or 100 μL of UP) was added to 0.5 mL of reaction medium and the mixture was incubated at 32°C. The reaction was terminated at 60 min with 400 μL of 24% (w/v) TCA. One milliliter of ammonium molybdate (0.7% [w/v] in 0.72 N H_2SO_4) and 50 μL of 0.1% (w/v) ascorbic acid were then added, and the A_{750} was determined after 10 min at room temperature. The Pi standards contained all assay ingredients except ATP and were incubated alongside the membrane vesicles.

Since our studies showed that PM ATPase activity in microsomal and UP membrane vesicles from tubers was relatively insensitive (average of 40% inhibition) to vanadate in the presence of specific inhibitors of mitochondrial and tonoplast ATPases and APases (azide, nitrate, and molybdate, respectively), microsomal membrane vesicles from potato leaf, stem, root, and tuber tissues were isolated (see above), and the relative sensitivities of their ATPases to vanadate were compared. Two 3-month-old, field-grown potato plants (cv Russet Burbank) provided the various tissues for this study. Whole plants (intact with roots and tubers) were harvested, soil was washed from the root systems, and leaflets of the fourth and fifth compound leaves (counting back from the shoot apices) were used. Stem tissue consisted of 10-cm-long sections, starting at 15 cm and extending down to 25 cm from the shoot apices. One-centimeter-thick sections of the apical portions of tubers (including periderm), as well as the entire root system from each plant, were used as additional sources of tissue. Tissues from the two plants were processed separately; therefore, each plant served as a replicate. The V_2O_5 solution was prepared in 20 mM NaOH (Gallagher and Leonard, 1982). The microsomal membranes were preincubated in reaction medium with inhibitors (azide, nitrate, molybdate, and vanadate, where indicated) for 15 min at 35°C prior to starting the reaction by adding ATP.

In addition to inhibitor studies, membrane marker enzymes were assayed to assess the extent of contamination of tuber PMs by non-PM-derived vesicles. GS II activity (a PM marker) was determined in microsomal and UP membrane fractions by modified methods of Widell and Larsson (1990). The reaction medium (100 μL) contained 330 mM Suc, 0.8 mM spermine, 16 mM cellobiose (Serva, Heidelberg, Germany), 4 mM EGTA, 4 mM $CaCl_2$, 0.5 mM DTT, 0.015% (w/v) digitonin, 0.5 mM UDP-Glc, and 476 pmol of [^{14}C]UDP-Glc (315 mCi/mmol; Amersham, Canada) in Hepes-KOH buffer (50 mM, pH 7.2), along with 25 μL of membrane suspension. All chemicals except [^{14}C]UDP-Glc and cellobiose were obtained from Sigma. The reaction was incubated at 25°C for 30 min and was terminated by adding 1 mL of 70% (v/v) ethanol, 50 μL of 50 mM $MgCl_2$, and 150 μL of boiled crude microsomal preparation. After settling overnight at 4°C, samples were centrifuged at 1640*g* for 15 min at room temperature. The supernatant was discarded and the pellet was washed four times with 2.5 mL of ethanol to effectively remove unreacted [^{14}C]UDP-Glc. The pellet was transfered to a scintillation vial and counted in 10 mL of ScintiVerse E (Fisher Scientific) with a liquid scintillation counter (Minaxiβ Tri-Carb 4000, Packard Instrument, Downers Grove, IL). GS II activity was expressed as μmol glucan formed mg^{-1} protein h^{-1}.

Latent IDPase activity (a Golgi marker) was assayed by the methods of Green (1988). The assay was carried out in 1 mL total volume containing 17 μg of membrane protein, 3 mM IDP, and 1 mM $MgCl_2$ in Tris buffer (50 mM, pH 7.5). Samples were incubated at 25°C for 60 min and the reaction was stopped by adding 400 μL of 24% (w/v) TCA. The Pi released from IDP hydrolysis was determined colorimetrically (Serrano et al., 1976). IDPase activity was assayed on the day of membrane isolation and, to account for enzyme latency, after 3 d of incubation at 4°C.

Cyt *c* reductase (which is insensitive to cyanide and antimycin A) activity (an ER marker) was assayed by the methods of Briskin et al. (1987). The reaction medium (1 mL) consisted of 2.5 mM NaCN, 180 μM Cyt *c* (Sigma), 150 μM NADH (Sigma), and 2 μM antimycin (Sigma) in sodium phosphate buffer (50 mM, pH 7.5), along with 300 μL of membrane suspension. The change in absorbance due to reduction of Cyt *c* was plotted at 550 nm and activity was quantified using the extinction coefficient of 28 mM^{-1} cm^{-1}. Enzyme activity was expressed as μmol Cyt *c* reduced min^{-1} mg^{-1} protein.

The pH optimum of PM ATPase was determined by coupling ATP hydrolysis to NADH oxidation via PK and LDH (Auffret and Hanke, 1981). The reaction medium (1 mL) contained 1 mg mL^{-1} LPC, 2 mM ATP, 5 mM PEP, 5 mM $MgCl_2$, 5 mM NaN_3, 100 mM KNO_3, and 1 mM Na_2MoO_4, 30 units each of PK and LDH, and 100 μL of membrane suspension in sodium acetate/Mes/Pipes/bis-Tris-propane (1,3-bis[Tris(hydroxy methyl)-methylamino] propane) buffer (10 mM each, pH 4.5–9.0). The reaction was initiated by adding 125 μM NADH (final concentration). ATPase kinetic assays were run (as above) with 0 to 2000 μM ATP (vanadium-free) in Pipes buffer (10 mM, pH 6.5). Oxidation of NADH was monitored at 340 nm and was quantified with the extinction coefficient of 6.22 μmol^{-1} cm^{-1}. K_m values and maximum rates were derived from equations describing first-order kinetics using the Fig. P program (Biosoft, Ferguson, MO).

Glutathione Determinations

Tissue from the apical portion of tubers (5 g fresh weight) was homogenized in a Polytron (Brinkmann) with 10 mL of 5% (w/v) 5-sulfosalicylic acid and centrifuged at 30,000*g* for 30 min. All manipulations were at 4°C. The supernatant was stored at −20°C under nitrogen. After thawing, 1.1 mL of supernatant was neutralized with 1.6 mL of 500 mM potassium phosphate buffer (pH 7.5) (Smith, 1985) and total glutathione was determined in 200 μL. Ten microliters of triethanolamine and 40 μL of 2-vinylpyridine were added to the remaining 2.5 mL, and the solution was incubated for 1 h at 23°C. GSSG was determined in 1 mL of this incubation mixture. GSH and GSSG were assayed by

the 5,5′-dithio-bis(2-nitrobenzoic acid)-GSSG reductase recycling method of Anderson (1985). Quantification of glutathione levels was based on a GSH standard curve.

GRase and GTase Assays

Tissue from the apical part of tubers (10 g fresh weight) was homogenized (with a pestle and mortar) in 20 mL of degassed potassium phosphate buffer (50 mM, pH 7.0) containing 0.5 mM PMSF and 5 mM DTT. The homogenate was passed through a layer of Miracloth and centrifuged at 30,000*g* for 30 min. All manipulations were at 4°C. The supernatant was stored at −20°C and thawed before use. The reaction medium (2 mL) for GRase activity consisted of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 2 mM GSSG, and 150 μM NADPH (final concentrations). The rate of oxidation of NADPH by enzyme extract was quantified at 340 nm using an extinction coefficient of 6.2 mM^{-1} cm^{-1} (Cakmak and Marschner, 1992; Rao et al., 1995).

GTase activity was determined in a total volume of 1.0 mL, containing 100 mM potassium phosphate buffer (pH 6.5) and 2 mM each of GSH and 1-chloro-2,4-dinitrobenzene (final concentrations). The rate of formation of *S*-2,4-dinitrophenylglutathione (a GSH-1-chloro-2,4-dinitrobenzene conjugate) by enzyme extract was quantified at 340 nm using the extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig and Jacoby, 1981; Warholm et al., 1985).

SDS-PAGE and Immunodetection

PM proteins were resolved via 8% SDS-PAGE (Laemmli, 1970). Following electrophoretic transfer (100 V for 1 h) of proteins from PAGE onto nitrocellulose, the blot was incubated at room temperature with polyclonal antibody to *Arabidopsis thaliana* PM H^+-ATPase (Pardo and Serrano, 1989), which had been diluted 1:1000 with blocking buffer. The blot was developed according to the method of Surowy and Sussman (1986).

Proteins from the 30,000*g* supernatant and mitochondria from 6-, 18-, and 30-month-old tubers at 0 (directly from 4°C storage) and 10 d of sprouting were resolved by SDS-PAGE on 10% gels (Laemmli, 1970). Isolated mitochondria were washed with BSA-free buffer (Kumar and Knowles, 1996) and solubilized with 0.2% Triton X-100. Proteins were electroblotted to nitrocellulose as described by Luethy et al. (1993), and GRase was probed with rabbit anti-spinach leaf GRase (Tanaka et al., 1994) diluted 1:1000, and alkaline phosphatase-conjugated goat secondary antibody to rabbit IgG, diluted 1:2500 with blocking buffer. GTase was probed with anti-*Hyoscymus muticus* GTase (Bilang and Sturm, 1995) diluted 1:1000 with blocking buffer. Mitochondrial matrix proteins were also separated (12% SDS gel) and blotted for GRase following removal of submitochondrial membrane particles (Kumar and Knowles, 1996). Protein was determined by a modified Lowry method (Bensadoun and Weinstein, 1976).

Statistical Procedures

Randomized, complete block designs were used in the growth, inhibitor, tissue comparison, and enzyme activity studies reported herein. Treatments were factorially arranged. Data were subjected to analysis of variance and sums of squares were partitioned into single-degree-of-freedom contrasts where appropriate. Marker enzyme analysis involved triplicate samples, and means with associated SD values are reported.

RESULTS

Tuber Respiration and Sprout Vigor

Although 19-month-old tubers produced more than 9 times as many sprouts as 7-month-old tubers by 10 d, sprout vigor (dry weight per sprout) from younger tubers was 7 times greater than that from older tubers (Table I). Total sprout dry weight per tuber, however, was not significantly affected by tuber age. Nineteen-month-old tubers had a higher rate of respiration than 7-month-old tubers throughout the sprouting period. Consistent with previous results (Kumar and Knowles, 1996), both ages of tubers displayed a transitory increase in respiration during the initial 24 h of acclimation to room temperature (data not shown). Respiration rates gradually declined from the maximum at 24 h and from 5 to 10 d remained relatively constant, averaging 5.1 and 12.7 mL CO_2 kg^{-1} h^{-1} for the 7- and 19-month-old tubers, respectively. Thus, the age-related difference in respiration of tubers harvested for comparison of PM ATPase activity at 5 and 10 d of sprouting was at least 2.5-fold (Table I).

Characterization of PMs

Since liquid polymer two-phase systems have up to this time not been adapted for isolating PM vesicles from po-

Table I. *Effect of potato tuber age and sprouting on respiration of whole tubers*

Tuber respiration was monitored in the dark at 23°C and sprouts were harvested at 10 d. Tubers were selected for isolation of PMs and comparison of ATPase activities at 0, 5, and 10 d of sprouting (see Fig. 2).

Tuber Age	Sprouting Period	Tuber Respiration	Sprouts	Sprout Dry Wt	
months	*d*	*mL CO_2 k^{-1} h^{-1}*	*no./ tuber*	*mg/ tuber*	*mg/ sprout*
7	0	2.53	–[a]	–	–
	5	5.07	–	–	–
	10	4.69	3	106	35.2
19	0	8.26	–	–	–
	5	11.5	–	–	–
	10	14.5	28[b]	137	4.9[b]
Age[c]		0.01[d]			
Time		0.01			
Age × time		0.01			

[a] –, Data not taken during these time periods. [b] F values for difference between tuber ages was significant at the 0.01 level. [c] Source of variation. [d] Significance levels for indicated sources of variation.

Table II. *Contribution of mitochondrial, tonoplast, and PM ATPases and APase to total activity in microsomal and UP membrane vesicle fractions isolated from potato seed-tuber cortical tissue*

Activity was assayed by the colorimetric determination of Pi released from ATP hydrolysis. The reaction mixture (5 mM ATP, PEP, and $MgCl_2$; 0.1 mg LPC mL^{-1}; 24 units of PK; and membrane vesicles in 10 mM Pipes buffer, pH 6.5) was incubated at 32°C for 1 h. Where indicated, NaN_3 (5 mM), KNO_3 (100 mM), Na_3VO_4 (100 μM), and $NaMoO_4$ (1 mM) were included in the reaction medium to inhibit mitochondrial, tonoplast, and PM ATPases and APases, respectively.

Source of Activity	Membrane Fraction	
	Microsomal	UP
	μmol Pi mg^{-1} protein h^{-1}	
No Inhibitors (NI)	57.1	55.4
NaN_3-sensitive (A)	9.3	2.9
KNO_3-sensitive (N)	15.8	7.8
$NaMoO_4$-sensitive (M)	4.7	4.8
PM ATPase NI-(A+N+M)	27.3	39.9
Na_3VO_4-sensitive	11.5	14.8
Membrane fraction (MF)[a]	NS[b]	
Inhibitors (I)	0.01	
NaN_3 vs NI × MF	0.01	
KNO_3 vs NI × MF	0.01	
$NaMoO_4$	NS	
PM ATPase vs NI × MF	0.01	
Na_3VO_4 vs NI × MF	0.01	
Na_3VO_4 vs PM ATPase × MF	0.01	

[a] Sources of variation. [b] Significance levels for indicated sources of variation.

tato tuber tissue, it was necessary to first characterize the ATPase and vesicles by utilizing suitable inhibitors, marker enzyme analysis, and immunoblotting. ATPase activity in microsomal and UP membrane vesicles was determined with and without inhibitors of mitochondrial, tonoplast, and PM ATPases and APase. In the absence of inhibitors, ATPase activity was comparable in microsomal and UP vesicles (Table II). The contribution to total activity by mitochondrial and tonoplast ATPases was 16 and 28%, respectively, in the microsomal vesicles. However, partitioning membranes into UP with the liquid polymer two-phase system significantly reduced contamination by mitochondrial and tonoplast ATPases to 5 and 14% of the control, respectively. Molybdate-sensitive APase activity in UP vesicles was minimal and equal to that in microsomal vesicles. When total activity was corrected for contamination by mitochondria, tonoplast, and APase, it was evident that PM ATPase activity had increased by 46% from the microsomal to the UP. Hence, PM ATPase accounted for 72% of total (noninhibited) activity in the UP, compared with only 48% in the microsomal fraction. Relative to activity in the absence of inhibitors, inhibition of microsomal ATPase by 100 μM vanadate was only 20%, but it increased marginally to 27% for vesicles partitioning into the UP. Even after contamination by non-PM-derived activity was accounted for, inhibition of PM ATPase by vanadate did not increase beyond 40% for the microsomal and UP vesicles (Table II). Since PM ATPase sensitivity to vanadate has been reported to be tissue-specific and modulated by various metabolites in other plant systems (Serrano, 1983; Stallaert et al., 1994), an additional study was conducted to compare the efficiency of vanadate inhibition of PM ATPase from different tissues of potato plants.

In the presence of specific inhibitors of non-PM-derived ATPases and APase, PM ATPase activity in microsomal vesicles from different tissues of potato plants exhibited differential sensitivity to V_2O_5 (Table III). Vanadate (100 μM) was much more effective at inhibiting PM ATPase activity in microsomes from leaf and stem tissues than in those from root and tuber tissues. These results cast considerable doubt on the relevance of vanadate inhibition as a diagnostic criterion for PM ATPase activity in potato tubers. The data in Table III also reveal that PM ATPase activities from etiolated root and tuber tissues were higher, on average, than those from leaf and stem tissues. SDS-PAGE of membrane protein from these tissues (Fig. 1) showed a 100-kD protein that cross-reacted with the antibody against *A. thaliana* PM ATPase (blot not shown). Relative to the other tissues, PM ATPase was greatly enriched as a proportion of microsomal protein in vesicles from tubers (Fig. 1).

Marker enzyme analysis further characterized and identified sources of contamination of microsomal and UP vesicles by non-PM-derived vesicles. GS II activity in tuber microsomes was comparable to that in leaf vesicles from the UP (Table IV). Moreover, GS II activity increased 3.2- and 2.5-fold when vesicles from leaves and tubers were partitioned to the UP, respectively. These results indicate that UP was more enriched in PM vesicles than the microsomal fractions and suggest that tuber microsomes are

Table III. *Differential vanadate sensitivity of PM ATPases in microsomal vesicles isolated from potato leaf, stem, root, and tuber tissue*

All assays were conducted in the presence of NaN_3, KNO_3, and $NaMoO_4$ (as specified in Table II). Where indicated (+), V_2O_5 was used at 100 μM (final concentration). Microsomal membranes were preincubated with the inhibitors for 15 min at 35°C and the reaction was started by adding ATP.

Tissue	V_2O_5	PM ATPase Activity	Inhibition
		μmol Pi mg^{-1} protein h^{-1}	%
Leaf (L)	−	7.46	–[a]
	+	1.85	75
Stem (S)	−	10.6	–
	+	3.99	63
Root (R)	−	60.6	–
	+	44.9	26
Tuber (T)	−	13.5	–
	+	8.77	35
		0.01[c]	–
Tissue[b]			
V_2O_5		0.01	–
Tissue × V_2O_5		0.01	–
L+S vs R+T		–	0.01
L vs S		–	0.01
R vs T		–	0.05

[a] –, No data or specific statistical comparison. [b] Sources of variation. [c] Significance levels for indicated sources of variation.

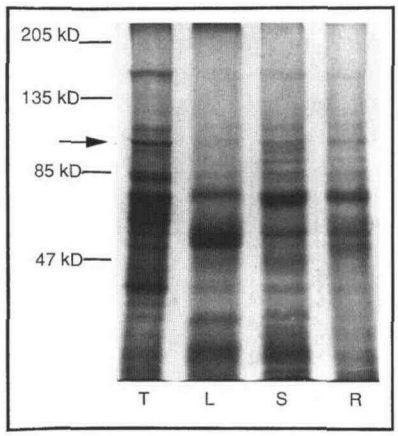

Figure 1. SDS-PAGE of protein from microsomal membrane vesicles from tuber (T), leaf (L), stem (S), and root (R) of 3-month-old, field-grown potato plants (58 μg protein/lane). The 100-kD polypeptide (arrow) cross-reacted with a PM H^+-ATPase antibody from *A. thaliana* (blot not shown).

inherently more enriched in PMs than leaf microsomes (as confirmed by the SDS-PAGE in Fig. 1). Antimycin-A-resistant Cyt *c* reductase activity (an ER marker) was undetectable in vesicles from the UP but present in microsomal fractions from both leaves and tubers, indicating that the two-phase system was effective in reducing ER contamination. Although differences in IDPase activity were evident between membrane fractions from leaves and tubers, activity increases were negligible over the 3-d incubation period at 4°C, reflecting an absence of latency and, thus, minimal contamination by Golgi vesicles. The pH optimum for enzyme activity was about 6.5, well within the range (pH 6.0–7.0) reported for plant PM ATPases (Sommarin et al., 1985; Sze, 1985; Palmgren and Sommarin, 1989).

Effects of Tuber Age and Sprouting on PM ATPase Activity

The effects of 0 (directly from a 4°C storage), 5, and 10 d of sprouting on PM ATPase activities in vesicles from 7- and 19-month-old seed-tubers are shown in Figure 2. The K_m of PM ATPase averaged 212 μM ATP and was not altered by either seed-tuber age or degree of sprout development. Activity was not affected by stage of sprouting; however, V_{max} of the ATPase from older tubers was 77% greater than that from younger tubers (averaged over stage of sprouting). This age-induced increase in activity cannot be attributed to a greater amount of enzyme per unit of PM protein, as evidenced by comparing the relative amounts of the 100-kD ATPase protein from PMs from young and old tubers in the gels and immunoblots of Figure 3. Densitometer analysis of the gels in Figure 3 revealed that younger tubers had 11% more PM ATPase per unit of PM protein than older tubers; however, per gram fresh weight, older tubers had 50% more microsomal protein than younger tubers. Since the amount of PM ATPase per unit of membrane protein was only slightly less (20% based on densitometry) in the microsomal fraction from 19-month-old tubers, compared with that from 7-month-old tubers (gels not shown), the older tubers likely contained more ATPase than younger tubers on a fresh weight basis.

Glutathione-Mediated Free Radical Scavenging

Glutathione levels and associated enzyme activities were determined in 6-, 18-, and 30-month-old tubers at 0, 5, 10, and 20 d of sprouting. Sprouting had no effect on the levels of GSH and GSSG or on GRase and GTase activities. Hence, data were averaged over the sprouting period. Total glutathione concentration increased linearly (r^2 = 0.99) at about 841 nmol per tuber per month (for an average 200-g tuber) as age advanced from 6 to 30 months (Table V). Similar trends were evident in both GSH and GSSG concentrations. Moreover, the proportion of GSSG to total glutathione remained relatively constant with age, indicating efficient turnover between oxidized and reduced pools.

Reduction of GSSG involves GRase, the activity of which also increased substantially with advancing tuber age (Table VI). The specific activity of GRase in 18- and 30-month-old tubers was 1.9- and 4.1-fold greater, respectively, than that in 6-month-old tubers during the 20-d sprouting interval. Similarly, GTase activity, which catabolizes and thus detoxifies lipid-hydroperoxides, also increased linearly with tuber age. Although the age-induced decline in soluble protein concentration depicted in Table VI is consistent with previous results (Kumar and Knowles, 1993c), the enhanced specific activities of GRase and GTase were not simply a consequence of declining protein content, since tuber age also effected substantially higher activities on a fresh weight basis. SDS-PAGE and western blots showed that GRase (58 kD) increased per unit of tuber protein with advancing tuber age (Fig. 4). Hence, the increased activity was likely due to more GRase produced in tubers as a consequence of aging. No such increases in cytosolic levels of GTase (25 kD) were evident with advancing tuber age (Fig. 4), suggesting that the age-enhanced activity of this enzyme may be due to a positive effector, increased substrate availability, or both. GRase and GTase increased as a proportion of total mitochondrial protein as tuber age advanced from 6 to 18 months (Fig. 5) but then remained relatively constant through 30 months (data not shown). The age-induced increase in GRase was even more apparent in gels and western blots of mitochondrial matrix protein (Fig. 6). Further studies characterizing the effect of tuber age on GRase and GTase isozymes in cytosolic and mitochondrial fractions are warranted.

DISCUSSION

Older tubers characteristically establish a higher rate of respiration than younger tubers during the initial stages of sprouting and maintain this higher rate through at least 30 d (Kumar and Knowles, 1993c). This age-induced increase in respiration results in more ATP production through a fully coupled, Cyt-mediated respiratory pathway (Kumar and Knowles, 1996). However, this increased ability of older tubers to generate ATP does not directly benefit sprout growth, as evidenced by equivalent total sprout weights and loss of vigor on a per

Table IV. *Marker enzyme analysis of membrane vesicle fractions isolated from tuber and leaf tissues of potato*

Membrane fractions were isolated with a two-step PEG-DEX (5.8% w/w) two-phase system. GS II activity was assayed by determining the synthesis of glucan from [^{14}C]UDP-Glc. Antimycin-A-insensitive Cyt *c* reductase (Cyt CR) activity was determined as the rate of reduction of Cyt *c* at 550 nm. IDPase activity was assayed by quantifying Pi released from IDP at time zero (0 d) and after 3 d of incubation at 4°C to indicate latency. Enzyme activities are the average ± SD of three determinations.

Tissue	Membrane Fraction	GS II[a]	Cyt CR[b]	IDPase Activity[c]	
				0 d	3 d
Tuber	Microsomal	264 ± 30	16.8 ± 4.4	14.7 ± 0.9	15.4 ± 0.5
	UP	655 ± 61	n.d.[d]	3.59 ± 0.14	4.75 ± 0.18
Leaf	Microsomal	82.5 ± 6.8	14.2 ± 1.3	12.7 ± 1.3	13.2 ± 0.1
	UP	260 ± 16.5	n.d.	0.30 ± 0.37	0.54 ± 0.09

[a] nmol glucan mg^{-1} protein h^{-1}. [b] μmol Cyt *c* mg^{-1} protein min^{-1}. [c] μmol Pi mg^{-1} protein h^{-1}. [d] n.d., Not detected.

sprout basis (Table I). Higher rates of respiration and associated oxidative phosphorylation are likely a response to significantly lower AEC in older tubers during the first 5 d of sprouting (Kumar and Knowles, 1996). The AEC of older tubers increases, concomitantly with the establishment of the age-induced difference in respiration, to equal that of younger tubers and then remains unaffected by age through the remainder of sprouting. Hence, sprouting older tubers must respire at a higher rate than sprouting younger tubers to achieve the same AEC. This indicates that older tubers have a greater need for, and thus produce and consume more, ATP during sprouting than younger tubers.

An enhanced sink for ATP in sprouting older tubers may be the ATPase in PMs that have become progressively leaky with advanced age. Age-induced loss of membrane integrity is well established for potato (Knowles and Knowles, 1989). Since H^+-ATPases consume 25 to 50% of the ATP content of cells (Leshem et al., 1992) and function in maintaining electrochemical gradients and ionic balance, the leaky cell membranes of older tubers likely constitute a substantial sink for ATP, which, in the absence of increased oxidative phosphorylation (respiration), would effect reduced AEC. To link our previous results of age-enhanced membrane permeability with increased ATP catabolism (energy expenditure), a procedure for isolating PMs from tubers was developed, and the effects of age and sprouting on H^+-ATPase activities were compared.

Prior to this report procedures for the isolation and purification of PM vesicles from potato tuber tissue utilized Suc gradient centrifugation to separate contaminating vesicles from PMs (Demandre, 1975; Jolliot et al., 1976; Bagdasaryan et al., 1983; Fakhari and Hall, 1984). This study adapts and modifies a liquid polymer, two-phase system, originally developed by Iswari and Palta (1989) for foliar tissue, to isolate PMs from tubers. The PEG-DEX, two-phase partitioning system was effective in eliminating the contamination of PMs by mitochondrial, tonoplast-, and ER-derived vesicles (Tables II and IV). A 2.5-fold increase in GS II activity, pH optimum of 6.5, and appreciably lower contamination from ER and Golgi membranes (as indicated by lower Cyt *c* reductase and latent IDPase activities) indicated that the UP fraction was enriched in PMs (Table IV). The residual activities of mitochondrial and tonoplast ATPases and APases remaining in UP vesicles were effectively suppressed during our kinetic studies by routine incorporation of suitable inhibitors in the assay medium.

Whereas marker enzyme analysis and inhibitor studies indicated significant enrichment of PM vesicles in UP, PM ATPase activity remained relatively insensitive to vanadate (Table II). Recent literature has indicated that reliance on vanadate sensitivity as the main criterion for PM H^+-ATPase is questionable. Sensitivity of yeast PM H^+-ATPase to vanadate changed with an apparent covalent modification of the enzyme by Glc or acid media (Serrano, 1983; Eraso and Gancedo, 1987). Moreover, Berczi et al. (1989) suggested that vanadate-sensitive ATPase activity is present in non-PM-derived vesicles isolated from wheat and maize. Topophytic effects on vanadate sensitivity have also been documented. For example, the ATPase activity in PMs isolated from the basal portions of mung bean hypocotyls was relatively insensitive to vanadate when compared with that isolated from the remaining hypocotyl tissue (Stallaert et al., 1994). Venken et al. (1991) showed that the degree of sensitivity of the PM ATPase to vanadate changes during aging and senescence of oat leaves. Upon further study, we determined that PM ATPase activity in microsomal vesicles from root and tuber tissues was only half as sensitive to vanadate as that from leaf and stem tissues (Table III). Surprisingly, data on vanadate sensitivity of ATPase in vesicles from potato tuber tissue has not been published; however, the sensitivity of ATPase in vesicles from potato roots to vanadate was reported to be only 24% (McArthur and Knowles, 1993). Our results thus agree with those presented by Berczi et al. (1989) and contribute further evidence that vanadate sensitivity is not an accurate indication of PM ATPase in all plant tissues.

Microsomes from tubers contained substantially more PM ATPase (100 kD, Fig. 1) per unit protein than those from leaf, stem, or root tissue. Despite the higher amount of enzyme in tuber microsomes, specific activity was substantially lower than that in microsomes from roots (Table III). This indicates that tissue-specific pools of PM ATPase are differentially activated in potato. Tissue-dependent, differential activation of ATPase has also been reported in mung bean (Kasamo and Nouchi, 1987).

PM ATPase is an important regulatory enzyme in cellular events such as adaptation to cold stress (Iswari and Palta, 1989) and developmental changes (Sussman and Surowy, 1987). In this study aging caused a substantial in-

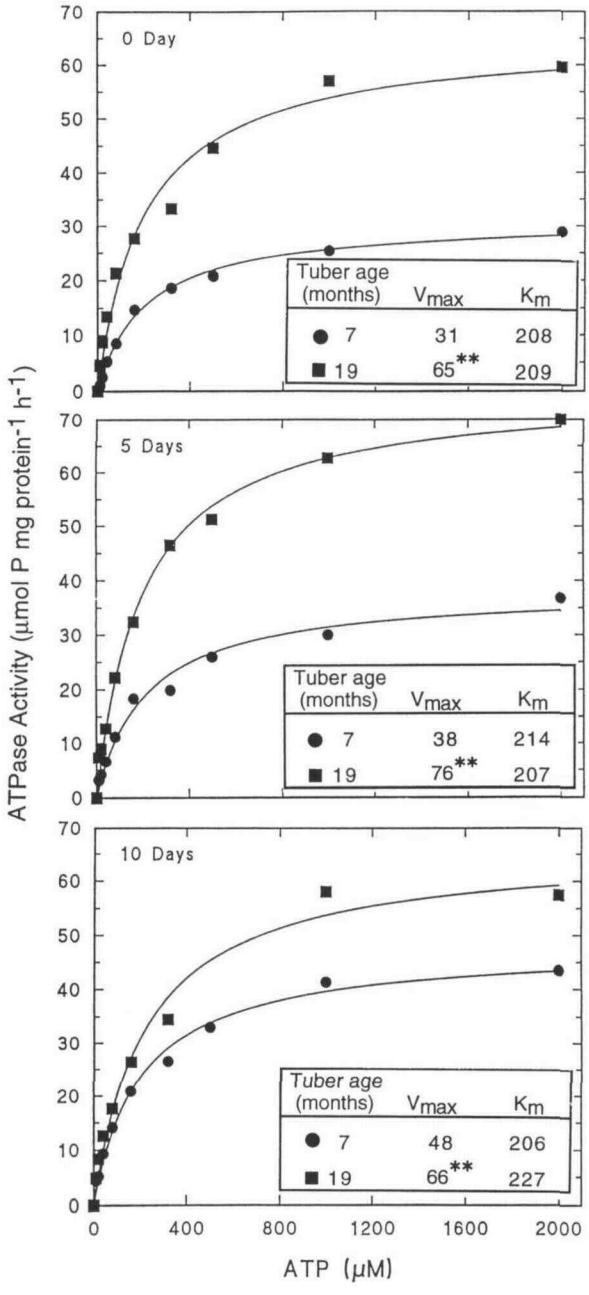

Figure 2. H^+-ATPase activity in PM vesicles from 7- and 19-month-old potato seed-tubers at 0 (directly from 4°C storage), 5, and 10 d of sprouting. Activity was determined by coupling ATP hydrolysis to NADH oxidation via PK and LDH in the presence of 5 mM NaN_3, 100 mM KNO_3, 1 mM $NaMoO_4$, and 1 mg mL^{-1} LPC at 23°C. **F value for comparison of V_{max} between ages was significant at the 0.01 level. Sprouting period and its interaction with tuber age did not affect V_{max} or K_m.

crease in PM ATPase activity, while the affinity of the enzyme for ATP remained unaffected (Fig. 2). The age-induced increase in specific activity was not due to a greater amount of PM ATPase per unit of membrane protein; younger tubers contained 11 and 20% more PM ATPase as a proportion of PM (Fig. 3) and microsomal membrane protein (data not shown), respectively, than older tubers. On a fresh weight basis, older tubers had 50% more microsomal protein than younger tubers (65 $\mu g/g$ fresh weight versus 97 $\mu g/g$ fresh weight). This observation, along with the fact that PM ATPase activity was greatly enhanced by tuber age, indicates that PM ATPase is a greater sink for ATP in older tubers.

Although this is the first report of an increase in PM H^+-ATPase activity caused by aging in plants, the biological phenomenon is not unique. Fernandes et al. (1988) compared Ca^{+2} Mg^{+2}-ATPase activities in young and old human erythrocytes and demonstrated similar results (increased V_{max}, no effect on K_m). Since ATPase is a membrane-bound enzyme, its activity is subject to modification by membrane architecture (Cooke and Burden, 1990). Changes in bilayer organization can effect configurational changes in membrane-bound enzymes, which may then alter kinetic properties (Carruthers and Melchior, 1986). In particular, ATPase activity can be altered by variation in lipid polar head group, fatty acid chain length, and degree of unsaturation (Sinesnsky et al., 1979; Palmgren et al., 1988; Palmgren and Sommarin, 1989; Cooke and Burden, 1990; Kasamo and Yamanishi, 1991). Direct interactions between active sites of the enzyme and hydrophobic environment of the PM (especially fatty acid acyl chains of phospholipids) are important in regulating ATPase activity (Kasamo, 1982, 1990).

Although the mechanism of activation and physiological significance of enhanced ATPase activity in aging tubers remains speculative, increased activity may be associated with changes in membrane structure that favor the gel phase. The age-induced decline in the double-bond index of phospholipids, which indicates increased saturation, along with increases in electrolyte leakage, are well established for potato (Knowles and Knowles, 1989). Moreover, ionophore-mediated breakdown of the electrochemical gradient across membranes activates ion pumping and

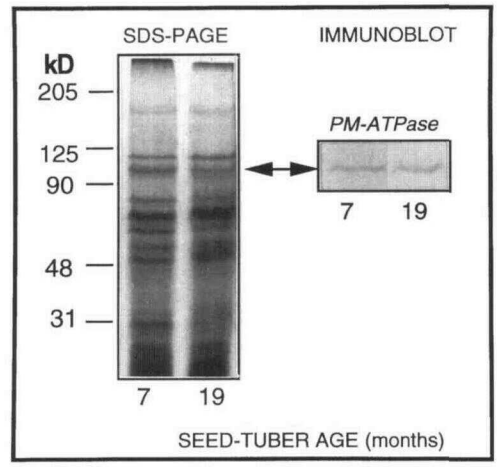

Figure 3. SDS-PAGE and immunoblots of protein from PM vesicles from 7- and 19-month-old potato tubers (82 μg protein/lane). Vesicles were isolated on a 5.8% (w/w) PEG-DEX two-phase system containing 15 mM NaCl. The 100-kD polypeptide cross-reacted with the PM H^+-ATPase antibody from *A. thaliana*.

Table V. *Effect of potato tuber age on levels of GSSG and GSH*

Glutathione levels of 6-, 18-, and 30-month-old tubers were averaged over 0, 5, 10, and 20 d of sprouting, since the effect of time was not significant.

Tuber Age	Glutathione			GSSG
	Total	GSH	GSSG	
month		*nmol g^{-1} fresh wt*		*% total*
6	79	75	3.8	4.9
18	134	127	6.6	4.9
30	180	169	10.9	6.1
LT[a]	0.01[b]	0.01	0.01	–[c]
Dev[d]	0.01	0.01	0.01	–

[a] LT, Linear trend. [b] Significance levels for indicated trends. [c] –, No data. [d] Dev, Deviations from linearity.

increased ATP hydrolysis (Sze, 1985). The precise mechanism by which ATPase is activated by advancing tuber age is beyond the scope of this paper; however, we speculate that the higher ATPase activity may be a primary physiological response to compensate for the age-induced breakdown in electrochemical gradient and ionic balance as a consequence of reduced membrane integrity.

Free-radical-driven lipid peroxidation plays a key role in the loss of membrane integrity in senescing plant tissues (Leshem, 1987; Gidrol et al., 1989) and during aging of potato tubers (Kumar and Knowles, 1993b). Peroxidation of membrane lipids yields lipid-hydroperoxides, which are highly reactive and damaging to cells. The initiation process of lipid peroxidation often involves hydroxyl radical formation by the Fe^{+2}-catalyzed decomposition of H_2O_2 (the Fenton reaction). Phospholipids of biological membranes are preferred targets for peroxidative damage since they contain polyunsaturated fatty acids (Thompson, 1988). Our previous studies demonstrated that ethane, malondialdehyde, and lipofuscin-like fluorescent compounds accumulate in tuber tissue with advancing age (Kumar and Knowles, 1993b). These compounds result from the decomposition of lipid-hydroperoxides and are thus regarded as sensitive markers of peroxidative damage (Fletcher et al., 1973; Konze and Elstner, 1978; Dhindsa et al., 1981). In addition, the activity of SOD, a substrate-inducible enzyme (Hassan and Scandalios, 1990) that catalyzes the dismutation of superoxide radicals to O_2 and H_2O_2, was much higher in older than in younger tubers, indicating that older tubers have a greater potential for accumulating the cytotoxic H_2O_2. POX and catalase activities also increased with tuber age, presumably to catabolize the H_2O_2 produced from increased SOD activity. These protective enzymes, however, cannot catabolize lipid-hydroperoxides directly. Another enzyme, GTase, which also exhibits POX activity (Ketterer et al., 1990; Bartling et al., 1993) and is inducible by a wide range of environmental stress conditions (Flury et al., 1995), efficiently catabolizes lipid-hydroperoxides that form during the propagation phase of lipid peroxidation (Haenen, 1989).

The glutathione-mediated free-radical-scavenging system plays an important role in containing lipid peroxidation (Foyer and Halliwell, 1976), and increased glutathione synthesis occurs in response to a number of stress conditions. For example, glutathione levels in the leaves of barley, tobacco, soybean, corn (Smith, 1985), and Arabidopsis suspension cultures (May and Leaver, 1993) increased 2- to 4-fold following exposure to oxidative stress imposed by elevated H_2O_2 levels. The degree of protection from oxidative stress was positively correlated with cellular glutathione content (May and Leaver, 1993). An increase in glutathione (GSH and GSSG) levels also occurred during aging of tubers (Table V), most likely in response to oxidative stress brought about by the age-induced increase in peroxidative damage.

Because of its hydrophilic nature, GSH cannot scavenge free radicals formed inside the lipid bilayer (Barclay, 1988). Peroxidized polyunsaturated fatty acids have to be deacylated first from the phospholipid-hydroxides by phospholipases to produce free lipid-hydroperoxides, which can then serve as substrates for GTase (McCay et al., 1976). Thus, phospholipase-catalyzed removal of lipid-hydrox-

Table VI. *Soluble protein content, GRase, and GTase activities of 6-, 18-, and 30-month-old tubers averaged over 0, 5, 10, and 20 d of sprouting (effect of time was not significant)*

GRase and GTase units are nmol of NADPH oxidized and *S*-2,4-dinitrophenyl-GSH formed per minute, respectively. Activities are expressed per milligram of protein and on a fresh weight basis.

Tuber Age	Soluble Protein	GRase		GTase	
months	*mg g^{-1} f wt*[a]	*units mg^{-1}*	*units g^{-1} f wt*	*units mg^{-1}*	*units g^{-1} f wt*
6	7.41	22.6	167	8.6	63.1
18	5.49	42.4	232	14.6	78.9
30	3.51	91.6	319	26.3	90.7
LT[b]	0.01[c]	0.01	0.01	0.01	0.01
Dev[d]	NS	0.01	NS	NS	NS

[a] f wt, Fresh weight. [b] LT, Linear trend. [c] Significance levels for indicated trends. [d] Dev, Deviations from linearity.

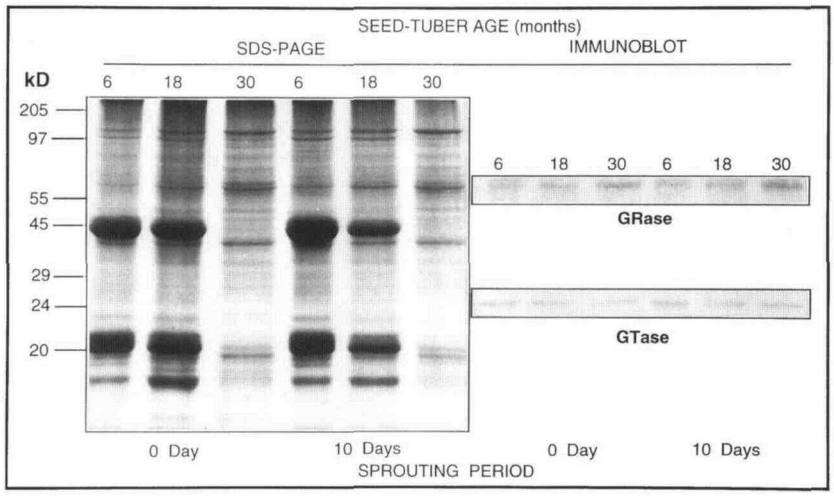

Figure 4. SDS-PAGE and immunoblots of soluble protein (30,000*g* supernatant) from 6-, 18-, and 30-month-old tubers at 0 (directly from 4°C storage) and 10 d of sprouting (27 μg protein/lane). The 58-kD polypeptide cross-reacted with anti-spinach leaf GRase and the 25-kD polypeptide cross-reacted with anti-*H. muticus* GTase.

ides and acyltransferase-mediated reconstruction of cell membranes are important strategies for maintaining membrane integrity (Haenen, 1989). The effect of seed-tuber age on acyltransferase activity remains to be established; however, GTase activity increases with tuber age (Table VI), most likely in response to increased availability of GSH and lipid-hydroperoxide substrates rather than to an increase in enzyme concentration (Fig. 4).

Despite the age-induced increase in GSH and GSSG concentrations, the proportion of GSSG to total glutathione remains constant with advancing tuber age, reflecting a concomitant increase in the ability of older tubers to reduce GSSG. This is facilitated by an age-dependent increase in GRase activity (Table VI), which maintains more than 90% of cellular glutathione in the reduced form (Tanaka et al., 1994) via the NADPH-dependent reduction of GSSG (Carlberg and Mannervik, 1985). The increased synthesis of GSH during aging and its regeneration from GSSG necessitate greater consumption of ATP (Hell and Bergmann, 1988) and NADPH, respectively, and thus constitute increased sinks for metabolic energy in older tubers. Moreover, enhanced activity of the glutathione-mediated free-radical-scavenging system indicates that oxidative stress is a central component of the aging process in tubers.

Age-enhanced, free-radical-mediated lipid peroxidation and increases in activities of enzymes that catabolize free radicals (SOD, POX, catalase, GTase) are not necessarily mutually exclusive processes. These enzymes are substrate-inducible (Bowler et al., 1989; Edreva et al., 1989; Flury et al., 1995), and higher activities are indicative of higher rates of production of superoxide and H_2O_2 in tissues. Similarly, in the absence of increased enzyme per unit of cytosolic protein, higher GTase activity reflects a greater availability of lipid-hydroperoxide substrate. In light of the age-induced increase in free-radical-scavenging ability, why then do older tubers show evidence of free-radical-mediated peroxidative damage (e.g. increases in ethane, malondialdehyde, lipofuscin-like fluorescent compounds, saturation of membrane phospholipids)?

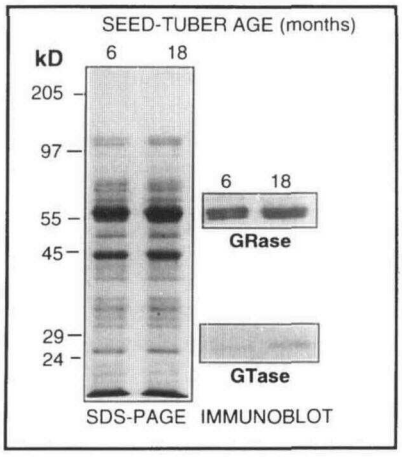

Figure 5. SDS-PAGE and immunoblots of mitochondrial protein from 6- and 18-month-old tubers at 10 d of sprouting. The 58- and 25-kD polypeptides cross-reacted with anti-GRase and GTase, respectively, as described in Figure 4 (27 μg protein/lane for SDS-PAGE and GRase blot; 54 μg protein/lane for GTase blot).

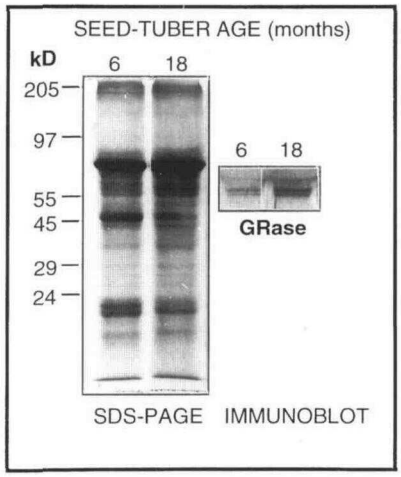

Figure 6. SDS-PAGE and immunoblots of GRase from the matrix of mitochondria from 6- and 18-month-old tubers at 10 d of sprouting (30 μg protein/lane). The 58-kD polypeptide cross-reacted with anti-spinach leaf GRase.

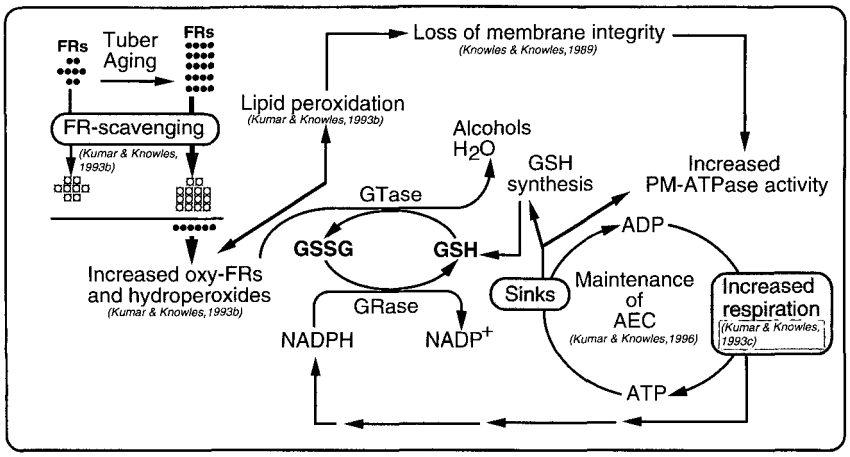

Figure 7. Schematic diagram illustrating possible associations among increased oxidative stress, loss of membrane integrity, and the higher respiration rates characteristic of older potato tubers during sprouting. A shift in the equilibrium between free radical (FR) production and consumption to favor production with advancing age is likely linked to the higher respiration of sprouting older tubers through enhancing two substantial "sinks" for metabolic energy: PM ATPase and GSH-mediated reduction of lipid-hydroperoxides and free radicals. References in parentheses document the indicated processes in aging tubers. See "Discussion" for further explanation. ● represents oxy-free radicals produced as a consequence of cellular metabolism; □ indicates free radicals that have been effectively quenched (neutralized) by various free-radical-scavenging systems.

A possible explanation is presented in Figure 7, which also links declining membrane integrity and glutathione-mediated free radical scavenging to the higher respiration and enhanced energy metabolism displayed by older tubers. In relatively young tubers, and under nonstressful conditions, free-radical-scavenging systems effectively quench free radicals produced as a consequence of normal metabolism. As tuber age advances, oxidative stress increases, and, in spite of concurrent increases in the ability to quench free radicals and the products of lipid peroxidation, the equilibrium between free radical production and removal (via the various free-radical-scavenging systems) gradually shifts in favor of production. The mechanism by which this equilibrium is disrupted is central to the aging phenomenon and remains unknown. However, the progressive increase in free radical production leads to even further lipid peroxidation, resulting in measurable increases in membrane permeability (reduced membrane integrity). At the cellular level, PM ATPase activity is stimulated (perhaps by an altered membrane architecture) and the higher activity partially compensates for the resulting disruption in ionic balance. PM ATPase in the membranes from older tubers thus becomes a greater sink for ATP relative to that from younger tubers. Additionally, glutathione is actively synthesized in response to the increasing oxidative stress and lipid-hydroperoxide accumulation. An age-induced increase in the rate of fully coupled, Cyt-mediated respiration allows older tubers to fuel increased GSH synthesis and PM ATPase activity (and any other age-enhanced sinks for ATP), while maintaining AEC at the same level as that characteristic of younger tubers. GTase and GRase activities also increase, resulting in enhanced reduction of lipid-hydroperoxides at the expense of NADPH. The pentose-P pathway is not stimulated by tuber aging (Sacher, 1980). Therefore, reducing equivalents for the age-enhanced GRase activity likely originate from the tricarboxylic acid cycle and involve NAD kinase (Dieter and Marme, 1984; Sauer and Robinson, 1985) and transhydrogenase (Carlenor et al., 1988). In fueling the increased energy demands of older tubers, higher respiration rates likely generate even more free radicals, further cascading the deteriorative process.

In summary, our results demonstrate that (a) PM ATPase is differentially activated in the various tissues of potato plants; (b) sensitivity of the enzyme to inhibition by vanadate depends on the origin of the potato tissue from which membrane vesicles are isolated (PM ATPase activity in vesicles from root and tuber tissues were relatively insensitive to vanadate compared with that from foliar tissues); (c) PM ATPase activity increases substantially with tuber age and thus is a greater sink for ATP in older tubers; (d) aging of tubers is accompanied by a progressive increase in oxidative stress, as evidenced by increased activity of the glutathione-mediated free-radical-scavenging system; and (e) age-induced synthesis of GSH and enhanced consumption of NADPH by increased GRase activity are comparatively greater sinks for metabolic energy (ATP and NADPH) in older tubers.

ACKNOWLEDGMENTS

We thank Professor R. Serrano (Departamento de Biotecnologia, Universidad Politecnica, Valencia, Spain) for graciously supplying PM H^+-ATPase antibody, K. Tanaka (Environmental Biology Division, National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan) for providing antibodies against GRase, and Arnd Sturm (Friedrich Miescher-Institut, Basel, Switzerland) for providing GTase antibody. We also thank N. Vasanthy (Lipid and Lipoprotein Research Group, University of Alberta, Edmonton, Canada) for helpful discussions and review of our manuscript.

Received May 15, 1996; accepted July 18, 1996.

LITERATURE CITED

Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol **113:** 549–555

Arrick BA, Nathan CF, Griffith OW, Cohn ZA (1982) Glutathione depletion sensitizes tumor cells to oxidative cytolysis. J Biochem **257:** 1231–1237

Auffret CA, Hanke DE (1981) Improved preparation and assay and some characteristics of Cl^--ATPase activity from *Limonium vulgare.* Biochim Biophys Acta **648:** 186–191

Bagdasaryan SG, Ladyzhenskaya EP, Lyubimova NP, Korableva NP, Metlitskii LV (1983) Cation stimulated ATPase activity of a plasma membrane preparation from potato tuber parenchyma. Prikl Biokhim Mikrobiol **18:** 554–561

Barber RF, Thompson JE (1980) Senescent dependent increase in the permeability of liposomes prepared from bean cotyledon membranes. J Exp Bot **31:** 1305–1313

Barclay LRC (1988) The cooperative antioxidant role of glutathione with lipid-soluble and water-soluble antioxidant during peroxidation of liposomes initiated in the aqueous phase and in the lipid phase. J Biol Chem **263:** 16138–16142

Bartling D, Radzio R, Steiner U, Weiler EW (1993) A glutathione S-transferase with glutathione peroxidase activity from *Arabidopsis thaliana.* Molecular cloning and functional characterization. Eur J Biochem **216:** 579–586

Bensadoun A, Weinstein D (1976) Assay of proteins in the presence of interfering materials. Anal Biochem **70:** 241–250

Berczi A, Larsson C, Widell S, Moller IM (1989) On the presence of inside-out plasma membrane vesicles and vanadate-inhibited K^+ Mg^{+2}-ATPase in microsomal fractions from wheat and maize roots. Physiol Plant **77:** 12–19

Bilang J, Sturm A (1995) Cloning and characterization of a glutathione *S*-transferase that can be photolabeled with 5-azidoindole-3-acetic acid. Plant Physiol **109:** 253–260

Bowler AT, Alliotte T, de Loose M, Montague MV, Inze D (1989) The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbagenifolia.* EMBO J **8:** 31–38

Briskin DP, Leonard RT, Hodges TK (1987) Isolation of plasma membrane: membrane markers and general principles. Methods Enzymol **148:** 5425–5458

Cakmak I, Marschner H (1992) Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol **98:** 1222–1227

Carlberg I, Mannervik B (1985) Glutathione reductase. Methods Enzymol **113:** 484–490

Carlenor E, Persson B, Glaser E, Anderson B, Rydstrom J (1988) On the presence of nicotinamide nucleotide transhydrogenase in mitochondria from potato tuber. Plant Physiol **88:** 303–308

Carruthers A, Melchior DL (1986) How bilayer lipids affect membrane protein activity. Trends Biochem Sci **11:** 331–335

Cooke DT, Burden RS (1990) Lipid modulation of plasmamembrane-bound ATPases. Physiol Plant **78:** 153–159

Demandre C (1978) Activites enzymatiques et composition lipidique de fractions enrichies en membranes cytoplasmiques isolees de tubercules de pommes de terre. Physiol Veg **13:** 103–114

De Vos CHR, Kraak LH, Bino RJ (1994) Ageing of tomato seeds involves glutathione oxidation. Physiol Plant **92:** 131–139

Dhindsa RS, Matowe W (1981) Drought tolerance in two mosses correlated with enzymatic defense against lipid peroxidation. J Exp Bot **32:** 79–91

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. J Exp Bot **32:** 93–101

Dieter P, Marme D (1984) A Ca^{+2}, calmodulin-dependent NAD kinase from corn is located in the outer mitochondrial membrane. J Biol Chem **259:** 184–189

Edreva A, Georgieva ID, Cholakova NI (1989) Pathogenic and non-pathogenic stress effects on peroxidases in leaves of tobacco. J Exp Bot **29:** 365–377

Eraso P, Gancedo C (1987) Activation of yeast plasma membrane ATPase by acid pH during growth. FEBS Lett **224:** 187–192

Fakhari H, Hall JL (1984) Changes in calcium-ATPase activity associated with the washing (ageing) of potato tuber discs. J Plant Physiol **117:** 69–79

Fernandes MAS, Tamagini GP, Pires EMV (1988) (Calcium and magnesium) ATPase and phosphoinositides of young and old human erythrocytes. Cienc Biol Mol Cell Biol **13**(3–4)**:** 71–84

Fletcher BL, Dillard CJ, Tappel AL (1973) Measurement of fluorescent lipid peroxidation products in biological systems. Anal Biochem **52:** 1–9

Flury T, Adam D, Kreuz K (1995) A 2,4-D-inducible glutathione S-transferase from soybean (*Glycine max*). Purification, characterization and induction. Physiol Plant **94:** 312–318

Foyer CH, Halliwell B (1976) The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta **133:** 21–25

Gallagher SR, Leonard RT (1982) Effect of vanadate, molybdate and azide on membrane-associated ATPase and soluble phosphatase activities of corn roots. Plant Physiol **70:** 1335–1340

Gidrol X, Serghini H, Noubhani A, Mocquot B, Mazalik P (1989) Biochemical changes induced by accelerated aging in sunflower seeds. I. Lipid peroxidation and membrane damage. Physiol Plant **76:** 591–597

Green JR (1988) The Golgi apparatus. *In* JL Hall, AL Moore, eds, Isolation of Membranes and Organelles from Plant Cells. Academic Press, New York, p 149

Guri A (1983) Variation in glutathione and ascorbic acid content among selected cultivars of *Phaseolus vulgaris* prior to and after exposure to ozone. Can J Plant Sci **63:** 733–737

Habig WH, Jacoby WB (1981) Assays for differentiation of glutathione *S*-transferase. Methods Enzymol **77:** 398–405

Haenen GRMM (1989) Thiols in oxidative stress. Some implications for catecholamine toxicity. PhD thesis. Free University, Amsterdam, The Netherlands

Hassan HM, Scandalios JG (1990) Superoxide dismutase in aerobic organisms. *In* RG Alscher, JR Cummings, eds, Stress Response in Plants: Adaptation and Acclimation Mechanisms. Wiley-Liss, New York, pp 175–200

Hell R, Bergmann L (1988) Glutathione synthetase in tobacco suspension cultures: catalytic properties and localization. Physiol Plant **72:** 70–76

Iswari S, Palta JP (1989) Plasma membrane ATPase activity following reversible and irreversible freezing injury. Plant Physiol **90:** 1088–1095

Jolliot A, Demandre C, Mazliak P (1976) Adenosine triphosphatases bound to plasmalemma mitochondria and microsomes or present in the cytoplasmic phase from potato tubers. Physiol Plant **38:** 287–292

Kasamo K (1982) Inhibition of membrane bound ATPase from *Nicotiana tabaccum* L. leaves with alkyltrimethylammonium bromide. Plant Cell Physiol **23:** 657–662

Kasamo K (1990) Mechanism for activation of plasma membrane H^+-ATPase from rice culture cells by molecular species of phospholipid. Plant Physiol **93:** 1049–1052

Kasamo K, Nouchi I (1987) The role of phospholipids in plasma membrane ATPase activity in *Vigna radiata* L. roots and hypocotyls. Plant Physiol **83:** 323–328

Kasamo K, Yamanishi H (1991) Functional reconstitution of plasma membrane H^+-ATPase from mung bean (*Vigna radiata* L.) hypocotyls prepared with various molecular species of phospholipids. Plant Cell Physiol **32:** 1219–1225

Ketterer B, Meyer DJ, Taylor JB, Pemble S, Coles B, Fraser G (1990) GSTs and protection against oxidative stress. *In* JD Hayes, CB Pickett, TJ Mantle, eds, Glutathione S-Transferases and Drug Resistance. Taylor and Francis, London, pp 97–109

Knowles NR, Knowles LO (1989) Correlations between electrolyte leakage and degree of saturation of polar lipids from aged potato (*Solanum tuberosum* L.) tuber tissue. Ann Bot **63:** 331–338

Konze JR, Elstner EF (1978) Ethane and ethylene formation by mitochondria as indication of aerobic lipid peroxidation in response to wounding of plant tissue. Biochim Biophys Acta **528:** 213–221

Kumar GNM, Knowles NR (1993a) Involvement of auxin in the loss of apical dominance and plant growth potential in aging potato seed-tubers. Can J Bot **71:** 541–550

Kumar GNM, Knowles NR (1993b) Changes in lipid peroxidation and lipolytic and free-radical scavenging enzyme activities during aging and sprouting of potato seed-tubers. Plant Physiol **102:** 115–124

Kumar GNM, Knowles NR (1993c) Age of potato seed-tubers influences protein synthesis during sprouting. Physiol Plant **89:** 262–270

Kumar GNM, Knowles NR (1996) Nature of enhanced respiration during sprouting of aged potato seed-tubers. Physiol Plant **97:** 228–236

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lee EH, Bennet JH (1982) Superoxide dismutase. A possible protective enzyme against ozone injury in snap beans (*Phaseolus vulgaris* L.). Plant Physiol **69:** 1444–1449

Leshem YY (1987) Membrane phospholipid catabolism and Ca^{+2} activity in control of senescence. Physiol Plant **69:** 551–559

Leshem YY, Shewfelt RL, Willmer CM, Pantoja O (1992) Plant Membranes: A Biophysical Approach to Structure, Development and Senescence. Kluwer, Dordrecht, The Netherlands, p 39

Luethy MH, Horak A, Elthon TE (1993) Monoclonal antibodies to the α- and β-subunits of the plant mitochondrial F_1-ATPase. Plant Physiol **101:** 931–937

Mauch F, Dudler R (1993) Differential induction of distinct glutathione S-transferases of wheat by xenobiotics and by pathogen attack. Plant Physiol **102:** 1193–1201

May MJ, Leaver CJ (1993) Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. Plant Physiol **103:** 621–627

McArthur DAJ, Knowles NR (1993) Influence of vesicular-arbuscular mycorrhizal fungi on the response of potato to phosphorous deficiency. Plant Physiol **101:** 3–14

McCay PB, Gibson DD, Fong KL, Hornbrook KR (1976) Effect of glutathione peroxidase activity on lipid peroxidation in biological membranes. Biochim Biophys Acta **431:** 459–468

Mikitzel LJ, Knowles NR (1990) The effect of potato seed-tuber age on plant establishment and amelioration of age linked effects with auxin. Plant Physiol **93:** 967–975

Palmgren GM, Sommarin M (1989) Lysophosphatidylcholine stimulates ATP dependent proton accumulation in isolated oat root plasma membrane vesicles. Plant Physiol **90:** 1009–1014

Palmgren GM, Sommarin M, Ulvskov P, Jorgensen (1988) Modulation of plasma membrane H^+-ATPase from oat roots by lysophosphotidyl choline, free fatty acids and phospholipase A_2. Plant Physiol **74:** 11–19

Pardo JM, Serrano R (1989) Structure of a plasma membrane H^+-ATPase gene from the plant *Arabidopsis thalina*. J Biol Chem **264:** 8557–8562

Pauls KP, Thompson JE (1981) Effect of *in vitro* treatment with ozone on the physical and chemical properties of membranes. Physiol Plant **53:** 255–262

Rao MV, Hale BA, Ormrod DP (1995) Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. Plant Physiol **109:** 421–432

Sacher RF (1980) Physiological age of potato seed tubers. Diss Abstr Int B Sci Eng **41:** 1999

Sauer A, Robinson DG (1985) Calmodulin dependent NAD-kinase is associated with both the outer and inner mitochondrial membranes in maize roots. Planta **166:** 227–233

Serrano R (1983) *In vivo* glucose activation of the yeast plasma membrane ATPase. FEBS Lett **156:** 11–14

Serrano R, Kanner BI, Racker E (1976) Purification and properties of the proton translocating adenosine triphosphatase complex of bovine heart mitochondria. J Biol Chem **251:** 2453–2461

Shinitzky M, Inbar M (1976) Microviscosity parameters and protein mobility in biological membranes. Biochim Biophys Acta **433:** 133–149

Sinesnsky M, Pinkerton F, Sutherland E, Simon FR (1979) Rate limitation of $(Na^+ + K^+)$ stimulated adenosinetriphosphatase by membrane acyl chain ordering. Proc Natl Acad Sci USA **76:** 4893–4897

Smith IK (1985) Stimulation of glutathione synthesis in photorespiring plants by catalase inhibitors. Plant Physiol **79:** 1044–1047

Smith IK, Kendall AC, Keys AJ, Turner JC, Lea PJ (1984) Increased levels of glutathione in a catalase-deficient mutant of barley (*Hordeum vulgare* L.). Plant Sci Lett **37:** 29–33

Sommarin M, Lundborg T, Kylin A (1985) Comparison of K^+, Mg^{+2} ATPases in purified plasma membrane from wheat and oat. Substrate specificities and effects of pH, temperature, and inhibitors. Plant Physiol **65:** 27–32

Stallaert VM, Cooke DT, Geuns JMC (1994) Isolation of plasma membranes from mung bean hypocotyl sections by two-phase partitioning: suitability of the technique for ageing tissues. Physiol Plant **90:** 671–678

Surowy TK, Sussman MR (1986) Immunological cross reactivity and inhibitor sensitivities of plasma membrane ATPase from plants and fungi. Biochim Biophys Acta **848:** 24–34

Sussman MR, Surowy TK (1987) Physiology and molecular biology of membrane ATPases. Oxford Surv Plant Mol Cell Biol **4:** 47–70

Sze H (1985) H^+-translocating ATPase: advances using membrane vesicles. Annu Rev Plant Physiol **36:** 175–208

Tanaka K, Sano T, Ishizuka K, Kitta K, Kawamura Y (1994) Comparison of properties of leaf and root glutathione reductases from spinach. Physiol Plant **91:** 353–358

Thompson JE (1988) The molecular basis for membrane deterioration during senescence. *In* LD Nooden, AC Leopold, eds, Senescence and Aging in Plants. Academic Press, New York, pp 51–83

Vasanthy N (1986) On the biochemical interactions of selenium with special reference to glutathione metabolism. PhD thesis. Indian Institute of Technology, Madras, India

Venken M, Asard H, Geuns MC, Caubergs R, de Greef Jan A (1991) Senescence of oat leaves: changes in the free-sterol composition and enzyme activities of the plasma membrane. Plant Sci **79:** 3–11

Walker MA, McKersie BD (1993) Role of ascorbate-glutathione antioxidant system in chilling resistance of tomato. J Plant Physiol **141:** 234–239

Warholm M, Guthenberg C, von Bahr C, Mannervik B (1985) Glutathione transferases from human liver. Methods Enzymol **113:** 499–504

Widell S, Larsson C (1990) A critical evaluation of markers used in plasma membrane purification. *In* C Larsson, IM Moller, eds, The Plant Plasma Membrane. Springer-Verlag, Heidelberg, Germany, pp 17–40