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Antioxidant Activities and Anticancer Cell Proliferation Properties of Wild Strawberries

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Abstract. **Fruit extracts from 17 to 18 representatives of three strawberry species [*Fragaria virginiana* Mill., *F. chiloensis* (L.) Mill., and *F.* ×*ananassa* Duchesne ex Rozier] were tested for the ability to inhibit proliferation of A549 human lung epithelial cancer cells. The fruit extracts also were tested for activities against free radicals, (peroxyl radicals, hydroxyl radicals, singlet oxygen, and superoxide radicals), the activities of antioxidant enzymes [glutathione peroxidase (EC 1.11.1.9), superoxide dismutase (EC 1.15.1.1), guaiacol peroxidase (EC 1.11.1.7), ascorbate peroxidase (EC 1.11.1.11), monodehydroascorbate reductase (EC 1.6.5.4), dehydroascorbate reductase (EC 1.8.5.1), and glutathione reductase (EC 1.6.4.2)], and the activities of nonenzyme antioxidant components, ascorbic acid and glutathione. Correlations between the proliferation of cancer cells and these antioxidant activities were calculated. At the species level, *F. virginiana* fruit extract inhibited the proliferation of A549 human lung epithelial cancer cells to a significantly greater extent (34% inhibition) than the extracts from fruit of either *F. chiloensis* (26%) or *F.* ×*ananassa* (25%) ($P < 0.0001$). Extracts from fruit of *F. virginiana* also had significantly greater antioxidant activities and higher activities of antioxidant enzymes and nonenzyme components than did extracts from the other two species. Among individual genotypes, there was a high positive correlation between antiproliferation of A549 cancer cells, antioxidant activities against free radicals, activities of antioxidant enzymes, and activities of nonenzyme components. Although all fruit extracts from all the strawberry genotypes inhibited proliferation of A594 cancer cells, fruit extracts from seven *F. virginiana* genotypes showed significantly greater antiproliferative effects than any of the *F.* ×*ananassa* or *F. chiloensis* genotypes. These genotypes, CFRA 0982, JP 95-1-1, NC 95-19–1, RH 30, NC 96-48-1, JP 95-9-6, and LH 50-4, may be especially useful in developing cultivars with greater anticancer potential.**

Reactive oxygen species, including peroxyl radicals (ROO·), hydroxyl radicals (·OH), superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), are generated as byproducts of normal metabolism. The accumulation of these reactive oxygen species or free radicals can cause oxidative damage to lipids, proteins, and nucleic acids, and thus promote cell death (Morel and Dangl, 1997; Satué-Gracia et al., 1997). Physiological defenses against oxidative stress involve small-molecule antioxidants and antioxidant proteins (Frei et al., 1992). Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting oxidizing chain reactions, and they play an important role as a health-protecting factor (Velioglu et al., 1998).

The antioxidant enzyme defense system consists of hundreds of different substances and mechanisms. Antioxidant enzymes have the capacity to lower the free radical burden and neutralize excess free radicals created by stress conditions. Antioxidant enzymes serve as catalysts that can affect one or more of the three stages of free radical formation: initiation, propagation, and termination. Therefore it is possible that antioxidant enzymes can prevent cellular and tissue damage in the human body (Baldwin, 1996; Bode and Dong, 2000).

Strawberries (*Fragaria* L.) are good sources of natural antioxidants (Heinonen et al., 1989; Wang et al., 2005). Previous studies showed that strawberry extracts exhibited high enzymatic activity for oxygen detoxification (Wang et al., 2005) and a high level of antioxidant capacity against free radical species including ROO·, ·OH, $O_2^{\cdot-}$, H_2O_2, and $^1O_2$ (Wang and Jiao, 2000; Wang and Lin, 2000). The main antioxidant enzymes in strawberry fruit are superoxide dismutase (SOD), guaiacol peroxidase (G-POD), glutathione peroxidase (GSH-POD), ascorbate peroxidase (AsA-POD), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), with ascorbic acid (AsA) and glutathione (GSH) as important nonenzyme components.

Strawberry extracts also exhibited chemopreventive and chemotherapeutic activities in vitro and in vivo (Carlton et al., 2001; Meyers et al., 2003; Wang et al., 2005), inhibited proliferation of the human lung epithelial cancer cell line A549, and decreased tetradecanoylphorbol-13-acetate (TPA)-induced neoplastic transformation of JB6 P^+ mouse epidermal cells (Wang et al., 2005). Pretreatment of JB6 P^+ mouse epidermal cells with strawberry extracts resulted in the inhibition of ultraviolet B (UVB)- and TPA-induced protein-1 (AP-1), and nuclear factor-κB (NF-κB) transactivation. Strawberry extracts

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also blocked TPA-induced phosphorylation of extracellular signal-regulated kinases (ERKs) and UVB-induced phosphorylation of ERKs and c-June amino terminal kinases (JNKs) in JB6 P^+ mouse epidermal cell culture (Wang et al., 2005).

AP-1 and NF-κB are transcription factors associated with carcinogenesis (Bode and Dong, 2000). AP-1 is composed of homodimers or heterodimers of the JUN and fructo-oligosaccharide (FOS) families (Angel and Karin, 1991). Many stimuli induce the binding of AP-1 to the promoter region of various genes that govern cellular processes such as inflammation, proliferation, and apoptosis (Baldwin, 1996). Inhibition of AP-1 activity has been shown to lead to suppression of cell transformation (Dong et al., 1997a). Some chemopreventive agents, including aspirin, tea polyphenols, and retinoic acid, have been reported to inhibit cell transformation and tumor promotion by suppressing AP-1 transactivation (Agadir et al., 1999; Dong et al., 1997a, b).

Nuclear factor-κB is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors. The NF-κB-regulated genes are part of the larger Rel/NF-κB family of transcription factors involved mainly in stress-induced, immune, and inflammatory responses. Nuclear factor-κB is also an important regulator in deciding cell fate, such as programmed cell death and proliferation control, and is critical in tumorigenesis (Baldwin, 1996). Reactive oxygen species (ROS) stimulate transcription by activating transcription factors such as AP-1 and NF-κB. Nuclear factor-κB and AP-1 signal transduction pathways are important in transformation and tumor promotion (Bode and Dong, 2000). TPA or UVB are carcinogens and can produce ROS and stimulate AP-1 and NF-κB activity by activating mitogen-activated protein kinase (MAPK) signaling pathways such as the ERK1/2, JNKs, p38, and MEK1/2 MAP kinases (Hou et al., 2004; Schulze-Osthoff et al., 1997).

JB6 P^+ mouse epidermal cells are sensitive to tumor promoter treatment and provide a cell culture-based model for studying the mechanism of tumor promotion (Hsu et al., 2000). Transformation-sensitive JB6 P^+ cells are preferred for probing early events in oxidative stress-related signaling leading to carcinogenesis, and for identifying the molecular targets for chemoprevention (Dhar et al., 2002). Antioxidants and extracts of apple peel have been shown to inhibit AP-1 activity in JB6 cells (Ding et al., 2004; Dong et al., 1997a, b). Therefore, some component or components of strawberry extract may be highly effective as a chemopreventive agent that acts by targeting the downregulation of AP-1 and NF-κB activities, blocking MAPK signaling, and suppressing cancer cell proliferation and transformation.

Although generally considered high, the levels of antioxidants and antioxidant capacity in strawberry extracts from whole fruit vary considerably among genotypes (Wang and Lin, 2000). This may be true partly because the cultivated strawberry (*F.* ×*ananassa*) is a hybrid of two very different wild species (Darrow, 1966). The paternal progenitor species, *F. virginiana*, is distributed throughout North America and was originally collected from the East Coast. The maternal progenitor species, *F. chiloensis*, is distributed on a thin strip of the western American coastline extending from the Aleutians through South America. Accessions of the progenitor species are valued by strawberry breeders as sources of novel traits, especially pest resistance and abiotic stress tolerance. Because strawberry is a relatively new crop, dating to the 1700s (Darrow, 1966), as few as three backcrosses can yield selections of cultivar quality (J.F. Hancock, pers. comm.). Therefore, if thought to be of value in improving antioxidant capacity and health-promoting qualities, accessions from these progenitor species could be readily incorporated into a strawberry breeding program.

Neither of the progenitor species has been evaluated for anticancer capability or activities of antioxidant enzymes and nonenzyme components. A core subset of the *Fragaria* collection maintained at the U.S. Department of Agriculture National Clonal Repository, Corvallis, OR, has been constructed to contain a group of native *F. virginiana* and *F. chiloensis* thought to be of value to strawberry improvement. This core subset is being characterized for many horticultural traits useful to breeders (Hancock et al., 2001a, b), including resistance to black root rot, common foliar diseases, and nematodes (Hancock et al., 2001b, 2002; Pinkerton and Finn, 2005).

The objectives of this study are 1) to identify wild strawberry genotypes with high anticancer and antioxidant activities for use in cultivar development, 2) to evaluate the anticancer capability and activities of antioxidant enzymes and nonenzyme components in representatives of the two progenitor species (*F. virginiana* and *F. chiloensis*) in comparison with representatives of the cultivated strawberry species (*F.* ×*ananassa*), and 3) to determine which antioxidant activities are more closely correlated with anticancer activities among representatives of these three species.

Materials and Methods

Chemicals. Ascorbate, chlorogenic acid, β-carotene, histidine, H_2O_2 (30% w/w), hydroxylamine hydrochloride, *N,N*-dimethyl-*p*-nitrosoaniline, xanthine, xanthine oxide, ascorbate oxidase, dithiothreitol (DTT), oxidized form of GSH (GSSG), GSH, GR, guaiacol, β-nicotinamide adenine dinucleotide (β-NADH, reduced form), β-nicotinamide adenine dinucleotide phosphate (β-NADPH, reduced form), nitro blue tetrazolium (NBT), resveratrol (3,5,4′-trihydroxystilbene), bovine serum albumin, Chelex 100, $FeSO_4$, and MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5,-diphenyl tetrazolium bromide] solution were purchased from Sigma Chemical Co. (St. Louis). 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), and α-tocopherol, and trichloroacetic acid were purchased from Aldrich (Milwaukee, WI). 2′, 2′ Azobis (2-amidinopropane) dihydrochloride was purchased from Wako Chemicals USA Inc. (Richmond, VA).

Strawberry genotypes. Eighteen genotypes of three strawberry species (*F. chiloensis*, *F. virginiana*, and *F.* ×*ananassa*) (Table 1) were used in this study. Most of the accessions of wild strawberry, and one *F.* ×*ananassa* accession, were chosen because they are included in the core subset described by Hancock et al. (2002). Others were chosen based on past knowledge of their ability to thrive and produce fruit at Beltsville, MD. Three *F. chiloensis* accessions, 12 *F. virginiana* accessions, and three *F.* ×*ananassa* accessions were selected. The *F.* ×*ananassa* cultivar, Allstar, was selected for its relatively low antioxidant capacity, whereas 'Ovation' was selected for its relatively high antioxidant capacity among cultivars from the U.S. Department of Agriculture, Agricultural Research Station strawberry breeding program at Beltsville, MD (Lewers et al., 2004).

Table 1. *Fragaria* (strawberry) cultivars, selections, and wild genotypes grown on the north farm of the Henry A. Wallace Agricultural Research Center at Beltsville, MD, and evaluated for inhibition of A594 human lung cancer cell proliferation, activities against free radicals, activities of antioxidant enzymes, and nonenzyme components.

Taxon	Plant introduction no.	Common name	Origin
F. ×*ananassa*	PI 551406	Allstar	Beltsville, MD
	PI 551929	CFRA 0638	Beltsville, MD
	PI 634800	Ovation	Beltsville, MD
F. chiloensis	PI 551735	CFRA 0368	near Juneau, AK
F. chiloensis ssp. *chiloensis* f. *patagonica*	PI 612317	2 TAP 4B	La Tapera, Aisen, Chile
F. chiloensis ssp. *pacifica*	PI 612488	CFRA 1267	British Columbia
F. virginiana	PI 612570	JP 95-1-1	Florida
	PI 612320	JP 95-9-6	Georgia
	PI 612495	LH 50-4	Montana
	PI 612325	NC 96-5-3	Jones County, NC
	PI 612323	NC 96-35-2	Greene County, AL
	PI 612324	NC 96-48-1	Chester County, SC
F. virginiana ssp. *glauca*	PI 552275	CFRA 0982	Near Ellsworth, ME
F. virginiana ssp. *grayana*	PI 612486	NC 95-19-1	Monroe County, MS
	PI 612569	NC 95-21-1	Pontotoc County, MS
F. virginiana ssp. *virginiana*	PI 612493	Frederick 9	Cochrane District, Ont., Canada
	PI 612497	Montreal River 10	Algoma District, Ont., Canada
	PI 612499	RH 30	Cook County, MN

Experimental design. The 18 strawberry genotypes were grown on the north farm of the Henry A. Wallace Agricultural Research Center at Beltsville, MD, using the advanced matted row production system (Black et al., 2002). Genotypes were randomly assigned to field plots in two blocks; each genotype was represented once in each block. In Summer 2005, plots were harvested individually. The fruit were hand harvested at a ripe stage; sorted to eliminate damaged, shriveled, and unripe fruit; and selected for uniform size and color. The fruit harvested from two plots of any genotype were kept separate for chemical analyses. From fruit of each plot, three subsamples were chemically analyzed separately, and means of the subsamples were combined to represent the fruit from each plot.

Fruit preparation. From fruit of each plot, undamaged berries were sorted randomly into three subsamples for separate analysis, frozen in liquid nitrogen, and then stored at –80 °C until they were used for assays. The three subsamples were analyzed separately. After chemical analysis, the means of the subsamples were combined to represent the fruit from each plot.

Free radical measurements

Assay for peroxyl radicals: Oxygen radical absorbance capacity (ORAC). Triplicate strawberries (5 g) from each plot of each genotype were extracted with 20 mL 80% acetone (containing 0.2% formic acid) using a Polytron (Brinkmann Instruments, Inc., Westbury, NY). The homogenized samples from the acetone extraction were then centrifuged at 14,000 g_n for 20 min at 4 °C. The supernatants were transferred to vials, stored at –80 °C, and later used for ORAC analysis after suitable dilution.

The ORAC assay was carried out using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system (Precision 2000; Bio-Tek Instrument, Winooski, VT) and a microplate fluorescence reader (FL ×800; Bio-Tek Instrument). Final ORAC values were calculated using the regression equation between Trolox concentration and the net area under the curve, and were expressed as millimoles Trolox equivalents (TE) per kilogram fresh weight (FW) (Huang et al., 2002).

Assay for superoxide radicals. Triplicate 100-g samples of strawberries from each plot of each genotype were pulverized and then centrifuged at 14,000 g_n for 20 min at 4 °C. The supernatants were transferred to vials stored at –80 °C until used for analysis after suitable dilution. The antioxidant activity of the strawberry extract against $O_2\cdot^-$ was determined using the methods of Richmond et al. (1981). The $O_2\cdot^-$ was generated by the xanthine xanthine–oxidase system. The antioxidant activity against the $O_2\cdot^-$ molecule was expressed as percentage inhibition of $O_2\cdot^-$ production in the presence of fruit extract, and was expressed as millimoles TE per kilogram FW.

Assay for hydroxyl radicals. Triplicate 100-g samples of strawberries from each plot of each genotype were pulverized and then centrifuged at 14,000 g_n for 20 min at 4 °C. The supernatants were transferred to vials stored at –80 °C until used for analysis after suitable dilution. The antioxidant activity of fruit extract against ·OH was determined using the methods of Richmond et al. (1981). The ·OH in aqueous media was generated through the Fenton reaction. Relative scavenging efficiency (percentage inhibition of hydroxylation) of fruit extract was estimated from the difference in absorbance [optical density (OD)] with and without the addition of fruit extract. The antioxidant activity of fruit extract against the ·OH molecule was expressed as millimoles TE per kilogram FW.

Assay for singlet oxygen. Triplicate 100-g samples of strawberries from each plot of each genotype were pulverized and then centrifuged at 14,000 g_n for 20 min at 4 °C. The supernatants were transferred to vials stored at –80 °C until used for analysis after suitable dilution. The production of $^1O_2$ by sodium hypochlorite and H_2O_2 was determined according to Chakraborty and Tripathy (1992) with minor modifications, in which *N*, *N*, dimethyl-*p* nitrosoaniline was used as a selective

scavenger of $^1O_2$ and histidine was used as a selective acceptor of $^1O_2$. The bleaching of *N, N*, dimethyl-*p*- nitrosoaniline was monitored spectrophotometrically at 440 nm. Relative scavenging efficiency (percentage inhibition production of $^1O_2$) of fruit extract was estimated from the difference in absorbance of *N, N*, dimethyl-*p*- nitrosoaniline with and without the addition of fruit extract. The antioxidant activity of fruit extract against the $^1O_2$ value was expressed as millimoles TE per kilogram FW.

Antioxidant enzyme measurements

Glutathione peroxidase, glutathione reductase, and superoxide dismutase determination. Triplicate fruit samples (10 g FW) from each plot of each genotype were homogenized in 10 mL 0.1 M Tris-HCl buffer (pH, 7.8) containing 2 mM EDTA-Na and 2 mM DTT. The homogenate was centrifuged at 20,000 g_n for 30 min at 4 °C, and the supernatant was used for the GSH-POD and GR assays after suitable dilution. The supernatant was further purified according to Wang et al. (1991) before assaying the SOD enzyme activity.

Glutathione peroxidase activity was determined using the method of Tappel (1978), with a slight modification. The reaction mixture contained 0.1 M Tris-HCl buffer (pH, 8.0), 0.4 mM EDTA, 1.0 mM NaN_3, 1.0 mM H_2O_2, 1.0 mM GSH, 0.15 mM NADPH, 1 U GR, and 100 µL enzyme extract. The total reaction volume was 1.0 mL. Hydrogen peroxide was added to start the reaction. Glutathione peroxidase activity was determined by the rate of NADPH oxidation at 340 nm via a spectrophotometer (Shimadzu UV-160A; Shimadzu Scientific Instruments, Columbia, MD). Enzyme activity was expressed as nanomoles NADPH oxidized per milligram protein per minute.

Glutathione reductase activity was assayed according to Smith et al. (1988). The activity of GR was determined by monitoring GSH-dependent oxidation of NADPH at 340 nm. The oxidized form of glutathione was added to start the reaction, and the rate of oxidation was calculated using the extinction coefficient of NADPH ($6.22 \cdot mm^{-1} \cdot cm^{-1}$). Glutathione reductase activity was expressed as nanomoles NADPH oxidized per milligram protein per minute.

Total SOD activity was assayed photochemically (Fridovich, 1986). Dicoumarol was included in the reaction mixture to inhibit reduction by pyridine nucleotide and to obtain a completely $O_2^{\cdot-}$-dependent reduction of NBT. One unit of SOD was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under assay conditions. Because inhibition is not linearly correlated with SOD concentration, a V/v transformation was used to obtain linearity, where V is the basic reaction rate without strawberry fruit extract and v is the reaction rate with extract. Linear correlation gave the equation

$$\text{SOD units} \cdot ml^{-1} = (0.459\ V/v - 0.032) \times \text{dilution factor}$$

The correlation coefficient for this line was 0.985.

Ascorbate peroxidase, guaiacol peroxidase, monodehydroascorbate reductase, and dehydroascorbate reductase determination. Triplicate fruit samples (10 g) from each plot of each genotype were pulverized in a cold mortar and pestle with 10 mL potassium phosphate buffer (0.1 M; pH, 7.3) containing 1 mM EDTA and 2 mM DTT. The homogenate was centrifuged at 12,000 g_n for 10 min at 4 °C. The supernatant was used for the AsA-POD, G-POD, MDAR, and DHAR assays after suitable dilution.

Ascorbate peroxidase activity was assayed according to the method of Amako et al. (1994). Hydrogen peroxide was added to start the reaction. Enzyme activity was expressed as nanomoles ascorbate oxidized per milligram protein per minute.

The G-POD assay mixture contained 0.1 M phosphate buffer (pH, 6.1), 4 mM guaiacol as donor, 3 mM H_2O_2 as substrate, and 1.0 mL crude enzyme extract. The total reaction volume was 3.0 mL. The rate of change in absorbance at 420 nm was measured, and the level of enzyme activity was expressed as the difference in absorbance (OD) per milligram protein per minute.

The DHAR activity was assayed by measuring the rate of NADPH oxidation at 340 nm. (Shigeoka et al., 1980). The reaction mixture contained 50 mM potassium phosphate, with a pH of 6.1, 0.2 mM NADPH, 2.5 mM dehydroascorbate, 2.5 mM GSH, 0.6 U GR from *Spinacia oleracea* L., and 0.1 mL diluted fruit extract (twofold dilution). The reaction was started by adding dehydroascorbate. Enzyme activity was expressed as nanomoles NADPH oxidized per milligram of protein per minute.

The MDAR activity was assayed by measuring the rate of NADH oxidation at 340 nm (Nakagawara and Sagisaka, 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH, 7.3), 0.2 mM NADH, 1.0 mM ascorbate, 1.0 U ascorbate oxidase, and 0.1 mL diluted fruit juice (twofold dilution) in a total volume of 1.0 mL. The reaction was started by adding ascorbate oxidase from *Cucurbita* L. (EC 1.10.3.3). Enzyme activity was expressed as nanomoles NADH oxidized per milligram protein per minute.

Ascorbic acid and glutathione determination. For measurement of AsA, triplicate fruit samples of 4 g from each plot of each genotype were pulverized and homogenized with a cold mortar and pestle using 8 mL ice-cooled 5% trichloroacetic acid. The homogenate was filtered through four layers of miracloth and centrifuged at 16,000 g_n for 10 min at 4 °C. The supernatant was used for the AsA assays. Ascorbic acid level was determined using the methods of Arakawa et al. (1981). A standard curve in the range 0 to 10 µmol AsA was used.

For measurement of GSH, triplicate strawberry fruit samples of 4 g were homogenized in 8.0 mL ice-cold, degassed 7.57 mM sodium ascorbate solution with chilled mortar and pestle under N_2 at 0 °C. The homogenate was filtered through four layers of miracloth and centrifuged at 30,000 g_n for 15 min at 0 °C. The supernatant was deproteinized in glass test tubes by incubation in a water bath at 100 °C for 3 min and then centrifuged at 15,000 g_n for 15 min at 0 °C. The supernatants were used for the GSH assay. Glutathione was assayed using the method described by Castillo and Greppin (1988).

MTT assay for inhibition of proliferation of cancer cells. MTT assay is a standard colorimetric assay for measuring cellular proliferation (cell growth). The amount of yellow MTT reduced to purple formazan is measured spectrophotometrically. This reduction takes place only when mitochondrial reductase enzymes are active, and thus conversion is directly related to the number of viable cells. For the assay to determine the level of inhibition of proliferation of the human lung epithelial cancer A549 cells (MTT assay), strawberry extracts were prepared by mixing 50 g strawberries with 50 mL distilled H_2O blended at high speed. The blended homogenates were strained, centrifuged at 6000 g_n at 4 °C for 20 min, and the supernatants were filtered. The supernatants were transferred to vials and stored at –80 °C until used for analysis after suitable dilution.

Human lung cancer cells, cell line A549, were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 50 U·mL^{-1} penicillin, and 50 μg·mL^{-1} streptomycin in 75 cm^2 T flasks at 37 °C, 85% humidity, and 5% CO_2 atmosphere. Subcultures were carried out every 2 to 3 d using a 0.25% trypsin and 0.02% EDTA solution. Briefly, A549 cells were plated in their growth medium at a density of 10^4 cells per well in 96-well flat-bottomed well cell culture plates and incubated at 37 °C. Twenty-four hours after plating, 0.33 μL strawberry extract (equal to 2.5 μg FW) was added to each well (except for control wells). After a 48-h incubation, 10 μL MTT solution was added in each well to form formazan salt crystals, and the plates were further incubated for 4 h. Then 100 μL solubilization solution [10% sodium dodecyl sulfate (SDS) in 0.01 M HCl] was added and the plate was incubated overnight at 37 °C. The amount of formazan produced was proportional to the number of viable cells (Mosmann, 1983). After incubation, the MTT–formazan was solubilized in 2-propanol and the OD was measured at a wavelength of 575 nm, with a reference wavelength of 690 nm, using a microplate spectrophotometer (Spectra MAX 250; Molecular Devices, Inc. Sunnyvale, CA), where higher OD values indicated more cell proliferation. Proliferation was expressed as a percentage of cell growth in wells that received no extract. Inhibition of proliferation was expressed as 100% minus the proliferation percentage.

Protein determination. The amount of total protein present in the fruit extract was estimated, according to Bradford (1976), for each of the enzyme assays. Bovine serum albumin, in a range from 0 to 30 μg, was used as a comparative standard. When the total amount of protein present in the fruit extract was determined, this amount was used to calculate the activity levels for each of the antioxidant enzymes.

Statistical analysis. To determine whether different strawberry genotypes had different inhibitory effects on cancer cell proliferation or different antioxidant activities, an analysis of variance (ANOVA) was done using SAS version 9.1.3 (SAS Institute, Cary, NC). The data were analyzed using the proc mixed command. The model in these analyses was that the observed variation for inhibition of cancer cell proliferation (MTT assay); antioxidant activities against ROO·, $O_2^{\cdot-}$, ·OH, and $^1O_2$ radicals; and antioxidant enzyme activities was a result of the genotype, and that the effects of the two blocks was random. Differences at $P < 0.05$ were considered significant.

To determine whether there may be general differences at the strawberry species level for inhibitory effects on cancer cell proliferation, or antioxidant activities, a second ANOVA used the model that observed variation was the result of species differences. Differences at $P < 0.05$ were considered significant.

A third ANOVA was done that was identical to the second, except data from only three *F. virginiana* genotypes were included to confirm the analysis was not biased as a result of having many more representatives of *F. virginiana* compared with only three representatives of either *F. chiloensis* or *F. ×ananassa*. The three genotypes selected were those with the lowest MTT assay means according to the first analysis: Montreal River 10, NC 96-35-2, and Frederick 9.

To determine which antioxidant activities against free radicals, antioxidant enzymes, and nonenzyme (AsA, GSH) components are more closely correlated with anticancer activity (cell viability, MTT uptake) among representatives of these three strawberry species, correlation coefficients were calculated using Excel 2003 (Microsoft Corp., Redmond, WA) and are reported as R^2 values.

RESULTS

Antioxidant activity. The scavenging capacities against ROO·, ·OH, $O_2^{\cdot-}$, and $^1O_2$ in various strawberry cultivars, selections, and wild genotypes are shown in Fig. 1. The scavenging capacity values against ROO· ranged from 27.98 to 61.69 TE per mmol·kg^{-1} FW. The *F. virginiana* ssp. *glauca* (S. Watson) Staudt accession from Maine, CFRA 0982 had the highest ROO· scavenging activity, followed by JP 95-1-1 [*F. virginiana* ssp. *grayana* (Vilm. ex J. Gay) Staudt], RH 30 (*F. virginiana* ssp. *virginiana*), NC 96-48-1 (*F. virginiana*), NC 96-35-2 (*F. virginiana*), JP 95-9-6 (*F. virginiana*), Montreal River 10 (*F. virginiana* ssp. *virginiana*), NC 95-19-1 (*F. virginiana* ssp. *grayana*), Frederick 9 (*F. virginiana* ssp. *virginiana*), LH 50-4 (*F. virginiana*), 2 TAP 4B [*F. chiloensis* ssp. *chiloensis* f. *patagonica* Staudt], NC 95-21-1 (*F. virginiana* ssp. *grayana*), CFRA 0368 (*F. chiloensis*), and CFRA 1267 (*F. chiloensis* ssp. *pacifica* Staudt). 'Allstar', CFRA 0638, and 'Ovation', the three *F. ×ananassa* genotypes, had the lowest scavenging capacities on ROO· radicals.

The scavenging capacity for ·OH ranged from 12.98 to 23.12 TE per mmol·kg^{-1} FW, reflecting a 1.78-fold difference among genotypes (Fig. 1). CFRA 0982 had the highest scavenging capacity for the ·OH free radical (23.12 TE per mmol·kg^{-1} FW). This equates to a 60.16% inhibition of ·OH activity. The percent inhibition of ·OH activity was determined by comparing berry fruit extract against ''blanks,'' which had been prepared similarly but without berry fruit extract. Meanwhile, 'Allstar' had the lowest ·OH scavenging efficiency with only 33.78% inhibition of ·OH activity. The scavenging capacity of these strawberry genotypes ranged from 7.01 to16.95 TE per mmol·kg^{-1} FW against $^1O_2$, and ranged from 3.32 to 11.79 TE per mmol·kg^{-1} FW against $O_2^{\cdot-}$ (Fig. 1). The relative scavenging efficiency (percentage inhibition of radical production) on $^1O_2$ and $O_2^{\cdot-}$ ranged from 18.87% to 45.63% and 27.45% to 81.54% respectively. CFRA 0982 had the best scavenging capacity for the reactive oxygen species not only for ROO· and ·OH, but also for $^1O_2$, and $O_2^{\cdot-}$, with 16.95 and 11.79 TE per mmol·kg^{-1} FW respectively for $^1O_2$, and $O_2^{\cdot-}$ (Fig. 1), which equates to relative scavenging efficiencies of 45.63% for $^1O_2$ and 81.54% for $O_2^{\cdot-}$. Meanwhile, 'Allstar' had the lowest ability to inhibit free radical activity for $^1O_2$, and $O_2^{\cdot-}$ with 7.01 and 3.32 TE per mmol·kg^{-1} FW respectively.

At the species level, extracts from fruit of *F. virginiana* had the highest scavenging oxygen species capacities compared with *F. chiloensis* and *F. ×ananassa* (Table 2). When the comparison was limited to the *F. virginiana* representatives lowest in inhibition of cancer cell proliferation (NC96-35-2, Frederick 9, and Montreal River 10) to balance the number of accessions representing each species, the scavenging capacity of *F. virginiana* extracts for all the free radicals tested was significantly greater than that of *F. chiloensis,* which was significantly greater than that of *F. ×ananassa* (Table 2).

Antioxidant enzymes and nonenzyme components. Different activities of antioxidant enzymes and content of nonenzyme components were detected in different strawberry genotypes (Figs. 2–4). CFRA 0982 had the highest activity of the peroxidase AsA-POD. CFRA 0982 and the *F. virginiana* ssp. *grayana* accession from Mississippi, NC 95-19-1 had the

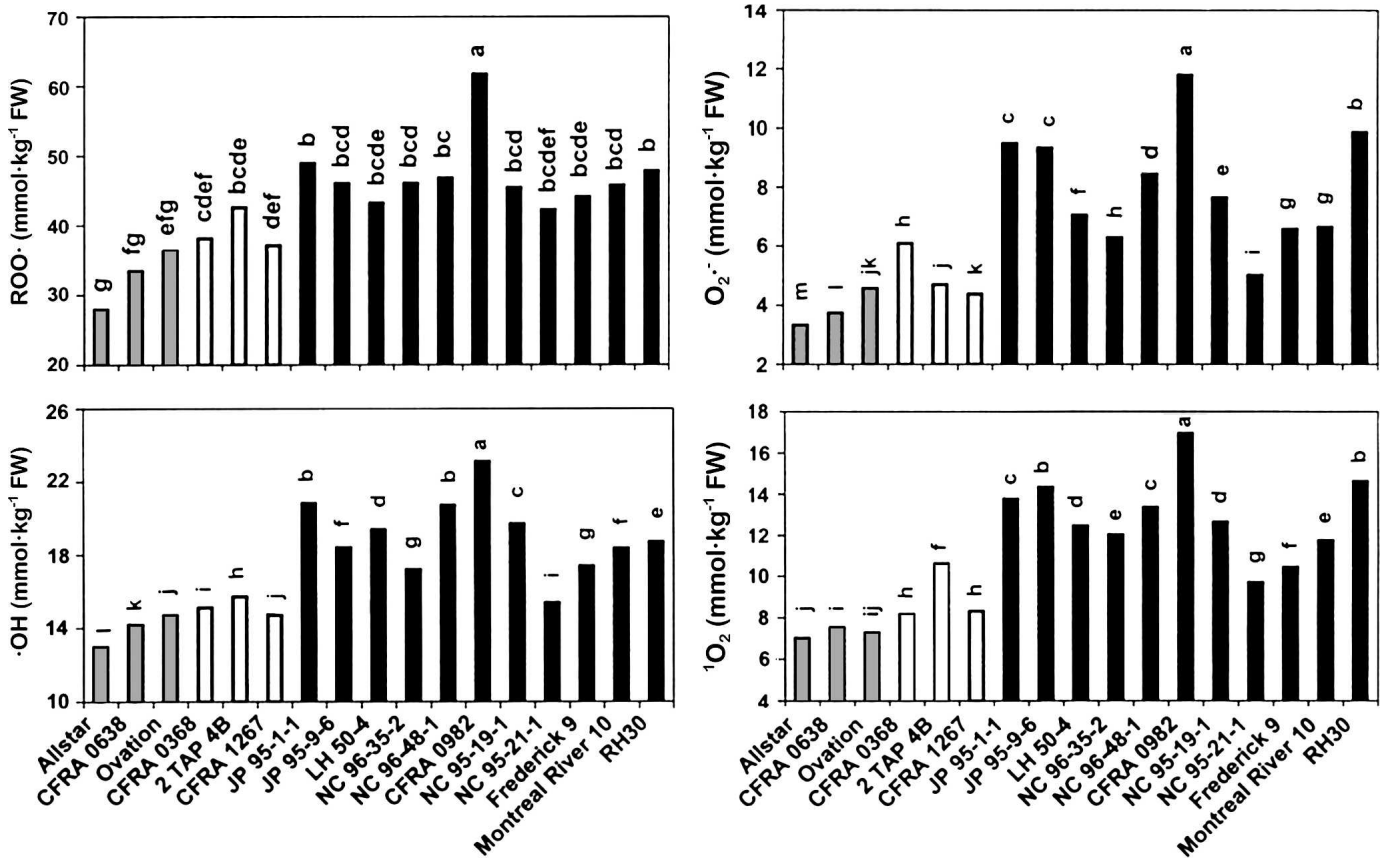

Fig. 1. The scavenging capacities against peroxyl radicals (ROO·), hydroxyl radicals (·OH), superoxide radicals ($O_2^{\cdot-}$), and singlet oxygen ($^1O_2$) in various *Fragaria* (strawberry) cultivars, selections, and wild genotypes. Gray bars denote *F.* ×*ananassa*, white bars denote *F. chiloensis*, and solid black bars denote *F. virginiana*. Data expressed as Trolox equivalents (TE) millimoles per kilogram fresh weight. Differences between genotypes were determined with an analysis of variance using SAS version 9.1.3 (SAS Institute, Cary, NC). The data were analyzed using the proc mixed command. Different letters indicate significant differences at $P \leq 0.05$.

highest levels of activity for the peroxidases G-POD and GSH-POD. In addition, NC 95-19-1 had the highest level of SOD followed by CFRA 0982. CFRA 0982 and the *F. virginiana* accession from Florida, JP 95-1-1, had the highest level of activity for the reductase GR. These two accessions plus the *F. virginiana* accession from North Carolina, NC96-5-3, had the highest level of activity for DHAR. JP 95-1-1 had the highest level of activity for MDAR, followed by CFRA 0982. CFRA 0982 and NC 95-19-1 had the highest levels of the nonenzyme component AsA, whereas CFRA 0982 and the *F. virginiana* accession from South Carolina, NC 96-48-1, had the highest levels of the nonenzyme component GSH. Meanwhile one or more of the *F.* ×*ananassa* genotypes had the lowest activities of all antioxidant enzymes and nonenzyme components, AsA and GSH (Figs. 2–4).

Among all species used in this study, extracts from fruit of *F. virginiana* had the highest activities of antioxidant enzymes (SOD, G-POD, GSH-POD, AsA-POD, MDAR, DHAR, and GR) and nonenzyme components (AsA and GSH) compared with *F. chiloensis* and *F.* ×*ananassa.* However, when only the three representatives of *F. virginiana* (NC 96-35-2, Frederick 9, and Montreal River 10, the *F. virginiana* accessions with the lowest percentage inhibition of A549 human lung epithelial cancer cells) were used in the comparison, the G-POD and DHAR activity levels of *F. virginiana* fruit extract were not significantly different from those of *F. chiloensis* (Table 2). When all representatives of the three species were compared, antioxidant enzyme and nonenzyme component activities were not significantly different between fruit extracts of *F. chiloensis* and *F.* ×*ananassa*. However, when only the three representatives of *F. virginiana* that were lowest in inhibition of cancer cell proliferation (NC 96-35-2, Frederick 9, and Montreal River 10) were used in the comparison, fruit extracts of *F.* ×*ananassa* were significantly lower in SOD, GSH-POD, MDAR, and GR activities.

Inhibition of proliferation of cancer cells. Antiproliferative activities of different genotypes of strawberry fruit extracts on the growth of human lung epithelial cancer A549 cells in vitro are summarized in Fig. 5. All genotypes of strawberry extracts showed inhibition of cancer cells proliferation. CFRA 0982 and JP 95-1-1 exhibited the greatest level of inhibition of A594 cancer cells, followed by NC 95-19-1, the *F. virginiana* ssp. *virginiana* RH 30, and NC 96-48-1. Fruit extracts from seven *F. virginiana* genotypes showed significantly greater antiproliferative effects than any of the *F.* ×*ananassa* or *F. chiloensis* genotypes. These genotypes are CFRA 0982, JP 95-1-1, NC 95-19-1, RH 30, NC 96-48-1, JP 95-9-6, and LH 50-4. The antiproliferative activities of strawberry fruit were expressed as MTT uptake, which was measured at a wavelength at 575 nm, where lower OD values indicate fewer cells and higher antiproliferative activities (Fig. 5).

At the species level, *F. virginiana* fruit extract inhibited the proliferation of A549 human lung epithelial cancer cells to a significantly higher extent (34% less than the uninhibited reference test) than the extracts from fruit of either *F. chiloensis*

Table 2. The inhibition of A549 human lung epithelial cancer cells (compared with 0.00 or 0% inhibition using a control with no extract added), scavenging capacities of reactive oxygen species, the activities of antioxidant enzymes and nonenzyme antioxidants in fruit extracts from representatives of three species of strawberries (*Fragaria virginiana*, *F. chiloensis*, and *F. ×ananassa*).

	Fragaria virginiana[z]	*F. chiloensis*[y]	*F. ×ananassa*[x]	F values[w]
Inhibition of A549 human lung epithelial cancer cells	0.34 a (0.30 a)	0.26 b	0.25 b	46.37**
Scavenging capacities of oxygen species				
Peroxyl radicals [TE (mmol·kg^{-1} FW)]	47.11 a (45.32 a)	39.26 b	32.60 c	17.86**
Hydroxyl radicals [TE (mmol·kg^{-1} FW)]	19.01 a (17.64 a)	15.18 b	13.95 b (c)	26.62**
Superoxide radicals [TE (mmol·kg^{-1} FW)]	8.02 a (6.51 a)	5.06 b	3.88 b (c)	19.22**
Singlet oxygen [TE (mmol·kg^{-1} FW)]	12.92 a (11.41 a)	9.04 b	7.28 b (c)	31.83**
Antioxidant enzymes				
Superoxide dismutases (U·mg^{-1} protein)	13.13 a (9.68 a)	8.21 b	6.71 b (c)	7.78**
Guaiacol peroxidase (ΔA·mg^{-1} protein per minute)	10.61 a (7.72 a)	6.83 b (ab)	5.96 b	6.05**
Glutathione peroxidase (nmol·mg^{-1} protein/min)	76.80 a (59.65 a)	45.37 b	38.09 b (c)	18.37**
Ascorbate peroxidase (nmol·mg^{-1} protein/min)	34.62 a (28.68 a)	17.00 b	17.41 b	27.44**
Monodehydroascorbate reductase (nmol·mg^{-1} protein/min)	38.39 a (30.45 a)	26.04 b	20.68 b (c)	12.49**
Dehydroascorbate reductase (nmol·mg^{-1} protein/min)	8.18 a (7.07 a)	5.95 b (ab)	5.00 b	10.50**
Glutathione reductase (nmol·mg^{-1} protein/min)	17.49 a (16.46 a)	11.20 b	9.16 b (c)	29.33**
Nonenzyme antioxidants				
Ascorbic acid (mmol·kg^{-1} FW)	2.49 a (2.37 a)	1.90 b	1.61 b	14.38**
Glutathione (μmol·kg^{-1} FW)	82.13 a (75.67 a)	5.42 b	1.40 b	16.80**

[z]Different letters in the same row indicate statistical significant differences at $P \leq 0.01$. Letters in parentheses indicate that the significance of the differences between species means changed when the analyses included only the three *F. virginiana* accessions with the lowest percentage inhibition of A549 human lung epithelial cancer cells: NC 96-35-2, Frederick 9, and Montreal River 10.

[y]Each value in this column (not in parentheses) is the mean value of *F. virginiana* representatives JP 95-1-1, JP 95-9-6, LH 50-4, NC 96-5-3, NC 96-35-2, NC 96-48–1, CFRA 0982, NC 95-19-1, NC 95-21-1, Frederick 9, Montreal River 10, and RH 30. Values in parentheses are means of NC 96-35-2, Frederick 9, and Montreal River 10, the *F. virginiana* accessions with the lowest percentage inhibition of A549 human lung epithelial cancer cells.

[x]Each value in this column is the mean value of CFRA 0368, 2 TAP 4B, and CFRA 1267.

[w]Each value in this column is mean value of 'Allstar', CFRA 0638, and 'Ovation'.

Differences between genotypes were determined with an analysis of variance using SAS version 9.1.3 (SAS Institute, Cary, NC). The data were analyzed using the proc mixed command.

**$P < 0.0001$.

ΔA, difference in absorbance between two readings; TE, Trolox equivalent.

(26%) or *F. ×ananassa* (25%; $P < 0.0001$). When only the three *F. virginiana* representatives with the least inhibitory effect were compared with the three representatives from the other two species, the *F. virginiana* fruit extracts still had a significantly greater inhibitory effect (30%). The difference in inhibitory effect between *F. chiloensis* and *F. ×ananassa* was not significant.

Correlations. In this study, positive correlations were observed between inhibition of A594 lung epithelial cancer cell proliferation (MTT assay) and all antioxidant-related chemical components tested, including activities against four free radicals, activities of seven antioxidant enzymes, and two nonenzyme components. The highest correlations between inhibition of A549 cancer cell proliferation and any of the antioxidant measures we tested were with the antioxidant enzyme AsA-POD ($R^2 = 0.947$), the activity against the free radical ·OH ($R^2 = 0.915$), and the antioxidant enzyme GR ($R^2 = 0.904$). Among both species and individual genotypes, there was a high positive correlation between inhibition of A549 cancer cell proliferation and antioxidant activities against free radicals, with R^2 values of 0.868, 0.884, 0.915, and 0.884 for ROO·, $O_2^{\cdot-}$, ·OH, and $^1O_2$ respectively (Table 3). A positive correlation also was found between cancer cell proliferation activity and activities of antioxidant enzymes, and nonenzyme components. The correlation values (R^2) between inhibition of cancer cell proliferation and SOD, G-POD, GSH-POD, ASH-POD, MDAR, DHAR, and GR were 0.745, 0.740, 0.875, 0.947, 0.882, 0.867, and 0.904 respectively (Table 3). The positive correlation values between inhibition of cancer cell proliferation and nonenzyme components were 0.708 and 0.755 for AsA and GSH respectively (Table 3).

Discussion

The results of this study showed that fruit of several wild strawberry accessions contain high antioxidants and have high antioxidant activities against ROO·, ·OH, $O_2^{\cdot-}$, and $^1O_2$. Different genotypes showed varying degrees of scavenging capacity on various radicals and among all the genotypes tested. CFRA 0982 had the highest scavenging capacity of active oxygen species. Free radicals are inherently unstable, because they are highly reactive and contain "extra" energy. To reduce their energy load, free radicals react with certain chemicals in the body, and in the process interfere with the cells' ability to function normally (Halliwell and Gutteridge, 1989). Antioxidants work in several ways to suppress free radicals: They may reduce the energy of the free radical, stop the free radical from forming in the first place, or interrupt an oxidizing chain reaction to minimize the damage incited by free radicals (Frei et al., 1992).

Natural antioxidant enzymes manufactured in the body provide an important defense against free radicals. Antioxidant enzymes exhibit synergistic interactions by protecting each other from specific free radical attacks (Blum and Fridovich,

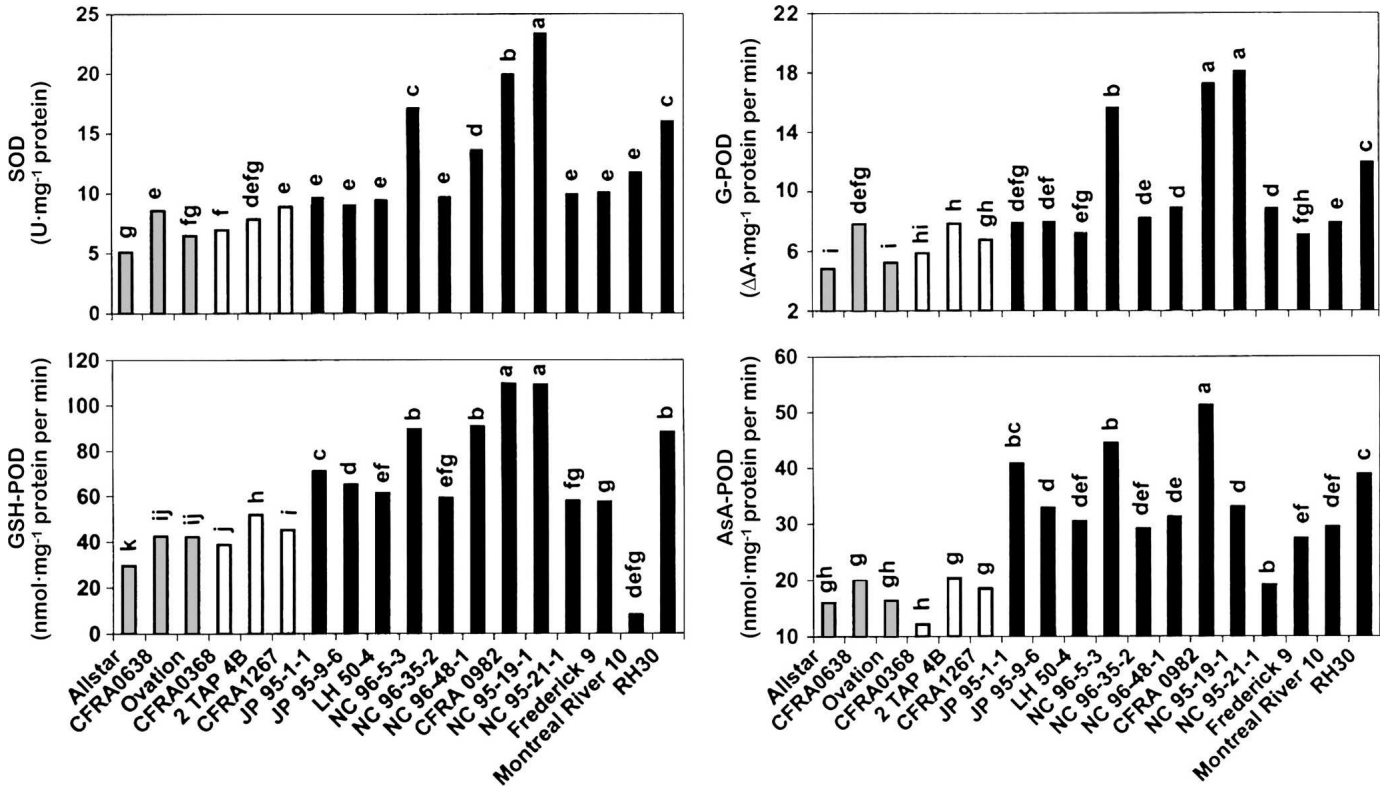

Fig. 2. Activities of antioxidant enzymes [superoxide dismutase (SOD), guaiacol peroxidase (G-POD), glutathione peroxidase (GSH-POD), and ascorbate peroxidase (AsA-POD)] in various *Fragaria* (strawberry) cultivars, selections, and wild genotypes. Gray bars denote *F.* ×*ananassa*, white bars denote *F. chiloensis*, and solid black bars denote *F. virginiana*. Differences between genotypes were determined with an analysis of variance using SAS version 9.1.3 (SAS Institute, Cary, NC). The data were analyzed using the proc mixed command. Different letters indicate significant differences at $P \leq 0.05$. ΔA, difference in absorbance between two readings.

1985), and they have the capacity to lower the free radical burden and neutralize excess free radicals created by stress conditions. Antioxidant enzymes serve as catalysts that can act at one or more of the three stages of free radical formation: initiation, propagation, and termination (Hong and Sporn, 1997). Therefore it is possible that antioxidant enzymes can prevent cellular and tissue damage in the human body. Glutathione peroxidase, GR, catalase, and SOD are among the most important antioxidant enzymes. The production of antioxidant enzymes in strawberry fruit is a complex process that is not yet totally understood. The antioxidant enzyme defense system consists of hundreds of different substances and mechanisms. The main antioxidant enzymes in strawberry fruit are SOD, G-POD, GSH-POD, AsA-POD, MDAR, DHAR, and GR. Different activities of antioxidant enzymes and content of nonenzyme components were detected in different strawberry genotypes (Figs. 2–4).

Superoxide dismutase activities in strawberry genotypes in this study range from 5.10 to 23.42 U·mg^{-1} protein. Superoxide dismutase is a primary antioxidant enzyme and catalyzes the breakdown of $O_2^{\cdot-}$ to O_2 and H_2O_2, removes $^1O_2$ as well as $O_2^{\cdot-}$, prevents formation of ·OH (Fridovich, 1986), and has been implicated as an essential defense against the potential toxicity of oxygen (McCord, 1979).

The activity of GSH-POD is correlated to antioxidant activity (Table 3). Guaiacol peroxidase is involved in a large number of biochemical and physiological processes and is abundant in cytosolic and mitochondrial compartments. The biological function of G-POD is to remove H_2O_2 and other hydroperoxides for protection against lipid peroxidation and DNA hydroperoxides (Chaudiere and Ferrari–Iliou, 1999). The mode of action of G-POD on the H_2O_2 substrate is to liberate free radicals that may be scavenged by catalase and GSH-POD.

Ascorbate peroxidase activity positively correlates with AsA and the scavenging capacity for ROO·, ·OH, $^1O_2$, and $O_2^{\cdot-}$ content in strawberry fruit extracts (Table 3). Ascorbate peroxidase is a heme-containing protein and is highly specific for ascorbate as the electron donor. The basic properties of AsA-POD are very different from those of G-POD with regard to its amino sequences and other molecular properties (Asada, 1992). Ascorbic acid serves as an excellent antioxidant and plays a fundamental role in the removal of H_2O_2 via the ascorbate–GSH cycle and produces dehydroxyascorbic acid. Dehydroxyascorbic acid is reduced to ascorbic acid by MDAR or DHAR at the expense of NADH and GSH (Halliwell and Gutteridge, 1989).

The correlation coefficient (R^2) for GR activities and scavenging capacities on oxygen species were also evident (Table 3). Genotypes with high activity of GR and other antioxidants enzymes were also found to have high antioxidant activity. Glutathione reductase is a ubiquitous NADPH-dependent enzyme and is present in cells of both plants and animals. The enzyme GSH-POD uses reduced GSH to eliminate H_2O_2: $2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$. Glutathione reductase then adds hydrogen ions to GSSG to regenerate GSH. A high GSH-to-GSSG ratio indicates a high level of GSH available for antioxidant activity. It has been suggested that in higher plants, GR may be a rate-limiting enzyme for defense against active O_2 toxicity (Gossett et al., 1996; Tanaka, 1994).

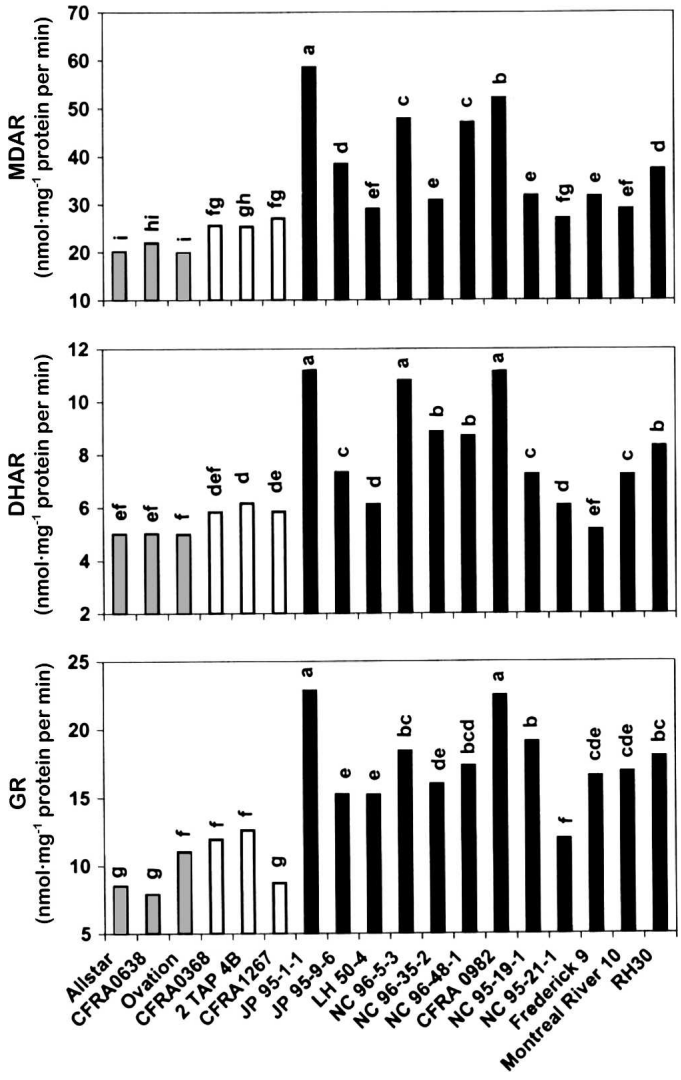

Fig. 3. Activities of antioxidant enzymes [monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR)] in various *Fragaria* (strawberry) cultivars, selections, and wild genotypes. Gray bars denote *F.* ×*ananassa*, white bars denote *F. chiloensis,* and solid black bars denote *F. virginiana*. Differences between genotypes were determined with an analysis of variance using SAS version 9.1.3 (SAS Institute, Cary, NC). The data were analyzed using the proc mixed command. Different letters indicate statistical significant differences at $P \leq 0.05$.

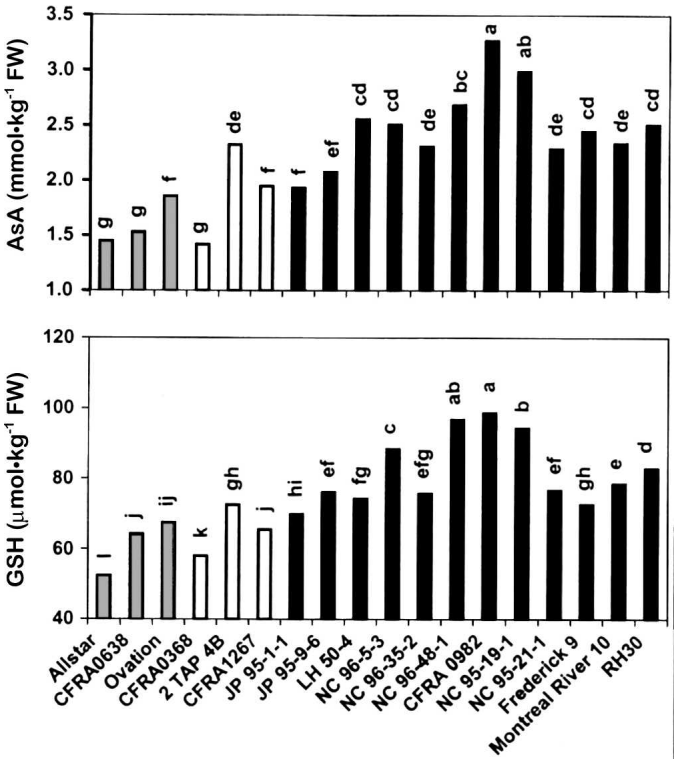

Fig. 4. The content of nonenzyme antioxidants [ascorbic acid (AsA) and reduced glutathione (GSH)] in various *Fragaria* (strawberry) cultivars, selections, and wild genotypes. Gray bars denote *F.* ×*ananassa*, white bars denote *F. chiloensis*, and solid black bars denote *F. virginiana*. Differences between genotypes were determined with an analysis of variance using SAS version 9.1.3 (SAS Institute, Cary, NC). The data were analyzed using the proc mixed command. Different letters indicate statistical significant differences at $P \leq 0.05$.

Antioxidants are notable for boosting the immune system because immune system cells in the bloodstream are so easily accessed by free radicals as well as by antioxidants. Antioxidants are molecules that can neutralize free radicals by accepting or donating an electron to eliminate the unpaired condition. Typically this means that the antioxidant molecule becomes a free radical in the process of neutralizing a free radical molecule to a nonfree radical molecule. Ascorbate can donate a hydrogen atom to a free radical molecule, thereby neutralizing the free radical while becoming an ascorbate radical itself. But the ascorbate radical is very stable because of its resonance structure. Moreover, ascorbate is readily regenerated from the ascorbate radical with NADH or NADPH-dependent reductases (Hossain and Asada, 1997). The correlations between scavenging capacities on ROO· (ORAC values) and AsA content, ·OH, $O_2^{\cdot-}$, and $^1O_2$ were also evident.

Glutathione is another important antioxidant. It readily interacts with free radicals, especially hydroxyl and carbon radicals, by donating a hydrogen atom. Reactions of this type provide protection by neutralizing reactive hydroxyl radicals. Glutathione plays an important role in the stabilization of many enzymes. It also serves as a substrate for DHAR and reacts directly with free radicals, including the hydroxyl radical, to prevent the inactivation of enzymes by oxidation of the essential thiol group. The majority of GSH in the cell is maintained in the reduced state (Kosower and Kosower, 1978). A high level of GSH is necessary for several physiological functions. This includes activation and inactivation of redox-dependent enzyme systems (Ziegler, 1985).

Antiproliferative activities of strawberry fruit extracts on the growth of human lung epithelial cancer A549 cells were different among the different genotypes. This is probably the result of different bioactive compounds in different genotypes. CFRA 0982 and JP 95-1-1 fruit extracts exhibited the highest antiproliferative activities on A549 cell growth. Although current literature about the origin and treatment of cancer is very complex and far from clear, many studies have documented the potential anticancer effects of flavonols and anthocyanins (Birt et al., 2001; Hou et al., 2003). Kang et al. (2003) reported that anthocyanins reduced the growth of human colon cancer cell lines HT-29 and HCT-116. Marko et al. (2004) also found that anthocyanidins significantly inhibited tumor cell growth. Strawberries are rich in anthocyanins,

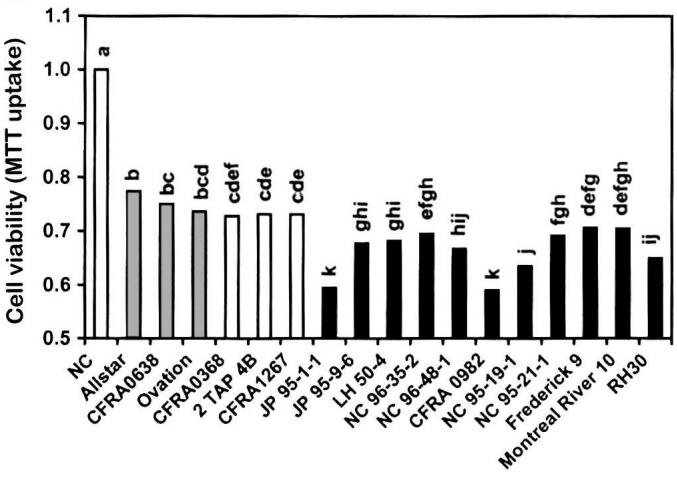

Fig. 5. Inhibition of human lung epithelial cancer A549 cells proliferation by extracts of various *Fragaria* (strawberry) cultivars, selections, and wild genotypes. A549 cells were plated at a density of 10^4 cells/well in the 96-well plate, cultured, differentiated, and treated with (2.5 µg/well FW) or without extract. After 48 h of incubation, 10 µL MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5,-diphenyl tetrazolium bromide] solution was added to each well to form formazan salt crystals, and the plates were further incubated for 4 h. Then 100 µL solubilization solution was added and the plate was incubated overnight at 37 °C. The optical density (OD) of the wells was measured at a wavelength of 575 nm, with a reference of 690 nm, using an ELISA plate reader. Results were calibrated with OD measured without cell culture. Gray bars denote *F.* ×*ananassa*, white bars denote *F. chiloensis*, solid black bars denote *F. virginiana*, and NC denotes no strawberry extract. Differences between genotypes were determined with an analysis of variance using SAS version 9.1.3 (SAS Institute, Cary, NC). The data were analyzed using the proc mixed command. Different letters indicate statistical significant differences at $P \leq 0.05$.

flavonoids, and phenolic acids (Heinonen et al., 1989). Their inhibitory activities may be the result of the synergistic combination of these polyphenols. Dietary freeze-dried strawberries were shown to effectively inhibit *N*-nitrosomethylbenzylamine-induced tumorigenesis in the rat esophagus (Evan and Vousden, 2001). Strawberry extracts have also been shown to inhibit benzo[a]pyrene-induced Syrian hamster embryo cellular transformation effectively (Xue et al., 2001). Several other kinds of fruit have also shown inhibition of hepatocellular carcinoma G_2 ($HepG_2$) human liver cancer cell proliferation (Carlton et al., 2001; Meyers et al., 2003, Sun et al., 2002).

Antioxidant activities were significantly correlated to antiproliferative activities on human lung epithelial cancer A549 cell growth (Table 3 and Fig. 5). It was suggested that antioxidants could prevent tumor initiation and act as a protective agent (Ding et al., 2004). Our data suggest that the activities of antioxidants and antioxidant enzymes may play an important role in the antiproliferative activity of strawberries, and the antioxidant activities are closely related with inhibition of A549 cell proliferation. The correlation data between antiproliferative effects and antioxidant activities from this study of three strawberry species further support previous finding of Wang et al. (2005). Similarly, Olsson et al. (2006) found high correlations between antiproliferation of breast cancer or prostate cancer cells and levels of ascorbate. However, Meyers et al. (2003) found no correlation between proliferation of liver cancer cells and total antioxidant content or antioxidant activity.

A multiplicity of antioxidants in strawberries, mediated by antioxidant enzymes, is beneficial because specific antioxidant molecules can be particularly effective for neutralizing specific reactive oxygen species. Of the biological free radicals, ·OH has the highest potential and is the most destructive (reactive). The radical $^1O_2$ has electrons in an excited state that react destructively with biomolecules containing double bonds. Anything that boosts the immune system is protective against cancer, but antioxidants have an additional anticarcinogenic effect through protection against DNA damage.

Although fruit extracts from all the tested strawberry genotypes inhibited proliferation of A594 cancer cells, fruit extracts from seven *F. virginiana* genotypes showed significantly

Table 3. Correlation coefficients (R^2) among A549 human epithelial lung cancer cell proliferation, antioxidant activities against free radicals, and activities of antioxidant enzymes and nonenzyme components from fruit extracts of three species of strawberry (*Fragaria virginiana*, *F. chiloensis*, and *F.* ×*ananassa*).

	MTT[z]	ROO·[y]	·OH[y]	O_2^{-}[y]	$^1O_2$[y]	SOD[x]	G-POD[x]	GSH-POD[x]	AsA-POD[x]	MDAR[x]	DHAR[x]	GR[x]	AsA[w]	GSH[w]
MTT	1.000													
ROO·	–0.868	1.000												
·OH	–0.915	0.911	1.000											
O_2·–	–0.884	0.896	0.921	1.000										
$^1O_2$	–0.884	0.925	0.932	0.945	1.000									
SOD	–0.745	0.677	0.707	0.652	0.678	1.000								
G-POD	–0.740	0.688	0.670	0.631	0.670	0.974	1.000							
GSH-POD	–0.875	0.830	0.882	0.825	0.854	0.942	0.904	1.000						
AsA-POD	–0.947	0.904	0.913	0.905	0.941	0.704	0.715	0.852	1.000					
MDAR	–0.882	0.799	0.864	0.870	0.825	0.491	0.462	0.702	0.843	1.000				
DHAR	–0.867	0.834	0.832	0.832	0.839	0.532	0.551	0.715	0.853	0.913	1.000			
GR	–0.904	0.892	0.936	0.895	0.885	0.659	0.631	0.822	0.887	0.854	0.853	1.000		
AsA	–0.708	0.812	0.793	0.655	0.772	0.829	0.792	0.880	0.757	0.498	0.529	0.707	1.000	
GSH	–0.755	0.812	0.823	0.716	0.794	0.857	0.813	0.941	0.763	0.595	0.615	0.713	0.927	1.000

[z]Cancer cell proliferation [MTT or 3-(4, 5-dimethylthiazol-2-yl)-2, 5,-diphenyl tetrazolium bromide] refers to the portion of cancer cell growth in comparison with growth when no strawberry fruit extract was added (1.00).
[y]Free radicals: peroxyl radicals (ROO·), hydroxyl radicals (·OH), superoxide radicals (O_2^{-}), singlet oxygen ($^1O_2$).
[x]Antioxidant enzymes: superoxide dismutase (SOD), guaiacol peroxidase (G-POD), glutathione–peroxidase (GSH-POD), ascorbate peroxidase (AsA-POD), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR).
[w]Antioxidant nonenzyme components: ascorbic acid (AsA) and glutathione (GSH).
R^2 values were calculated using Excel 2003 (Microsoft Corp., Redmond, WA).

greater antiproliferative effects than any of the *F.* ×*ananassa* or *F. chiloensis* genotypes. These genotypes, CFRA 0982, JP 95-1-1, NC 95-19-1, RH 30, NC 96-48-1, JP 95-9-6, and LH 50-4, may be especially useful in developing cultivars with greater anticancer potential. Of these genotypes, CFRA 0982, JP 95-1-1, NC 95-19-1, and RH 30 were found to be higher in antioxidant capacity (ORAC), total anthocyanins, and total phenolics than the cultivar Ovation (S.Y. Wang and K.S. Lewers, unpublished data).

The individual wild accessions highest in anticancer properties have other traits valued by strawberry breeders. NC 95-19-1 and JP 95-1-1 are valued as potential sources of resistance to anthracnose crown rot incited by *Colletotrichum fragariae* A.N. Brooks (K.S. Lewers, W.W. Turechek, S.C. Hokanson, J.L. Maas, J.F. Hancock, S. Serçe, and B.J. Smith, unpublished data). JP 95-1-1 is also resistant to crown rot incited by *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. [teleomorph *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk] (K.S. Lewers, W.W. Turechek, S.C. Hokanson, J.L. Maas, J.F. Hancock, S. Serçe, and B.J. Smith, unpublished data) and to both the northern root knot nematode (*Meloidogyne hapla* Chitwood) and the root lesion nematode [*Pratyenchus penetrans* (Cobb) Filipjev & Shuurmans Stekhoven] (Pinkerton and Finn, 2005). NC95-19-1, a female, is also resistant to the root knot nematode (Pinkerton and Finn, 2005) and leaf scorch (*Diplocarpon earlianum* Ellis & Everh.); tolerant to leaf blight [*Phomopsis obscurans* (Ellis & Everh.) Sutton] and powdery mildew [*Sphaerotheca macularis* (Wallr.:Fr.) Jacz. f.sp. *fragariae* Peries] (Hancock et al., 2001a, b, 2002); and has very good flavor (unpublished data). RH 30 is valued because it has multiple flowering cycles in a year (Hancock et al., 2001b), and is resistant to root knot nematode (Pinkerton and Finn, 2005), black root rot incited by unknown pathogens in Minnesota, leaf scorch, and leaf spot incited by *Mycosphaerella fragariae* (Tul.) Lindau (Hancock et al., 2001a).

CFRA 0982, displayed more health-promoting properties than all other genotypes tested. CFRA 0982 has other characteristics that make it a potentially valuable parent, including superior flavor that it passes to first-generation progeny (unpublished data) and multiple flowering cycles (Hancock et al., 2001b). CFRA 0982 is the only member of *F. virginiana* ssp. *glauca* tested in this study. It also is one of the few members of the *glauca* subspecies from the East Coast (in this case, the state of Maine). However, it is not included in the ''supercore'' core subset described by Hancock et al. (2002). This core subset includes four *F. virginiana* ssp. *glauca* representatives from the western United States and one from the Canadian province of Quebec. It may be useful to expand evaluation of wild strawberry genotypes beyond the core subset in addition to acquiring more representatives of East Coast *F. virginiana* ssp. *glauca.*

In conclusion, this study suggests that activities of antioxidants and antioxidant enzymes in strawberries are potential ingredients for cancer prevention. Our results also suggest that strawberries can inhibit human lung epithelial cancer A549 cell population growth. Different strawberry genotypes had different levels of antioxidant capacity, antioxidant enzyme activity, nonenzyme components, and antiproliferative activities on A549 cells, and the correlations between all of these measures were positive and high. Therefore, the inhibition of cancer cell proliferation may be the result of the synergistic combination of multiple polyphenols found in the berries. Dietary intake of strawberries with high antioxidant capacity will have the potential to reduce cancer risk and other diseases. This study clearly shows the potential value of wild strawberry accessions in the development of strawberry cultivars with improved health-promoting qualities.

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