

Tobacco Nectarin I

PURIFICATION AND CHARACTERIZATION AS A GERMIN-LIKE, MANGANESE SUPEROXIDE DISMUTASE IMPLICATED IN THE DEFENSE OF FLORAL REPRODUCTIVE TISSUES*

Received for publication, July 20, 2000, and in revised form, August 18, 2000
Published, JBC Papers in Press, August 21, 2000, DOI 10.1074/jbc.M006461200

Clay Carter and Robert W. Thornburg‡

From the Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011

Nectarin I, a protein that accumulates in the nectar of *Nicotiana* sp., was determined to contain superoxide dismutase activity by colorimetric and in-gel assays. This activity was found to be remarkably thermostable. Extended incubations at temperatures up to 90 °C did not diminish the superoxide dismutase activity of nectarin I. This attribute allowed nectarin I to be purified to homogeneity by heat denaturation of the other nectar proteins. By SDS-polyacrylamide gel electrophoresis, nectarin I appeared as a 29-kDa monomer. If the protein sample was not boiled prior to loading the gel, then nectarin I migrated as 165-kDa oligomeric protein. By matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, the protomer subunit was found to be a 22.5-kDa protein. Purified nectarin I contained 0.5 atoms of manganese/monomer, and the superoxide dismutase activity of nectarin I was not inhibited by either H₂O₂ or NaCN. Following denaturation, the superoxide dismutase activity was restored after Mn²⁺ addition. Addition of Fe²⁺, Cu²⁺, Zn²⁺, and Cu²⁺/Zn²⁺ did not restore superoxide dismutase activity. The quaternary structure of the reconstituted enzyme was examined, and only tetrameric and pentameric aggregates were enzymatically active. The reconstituted enzyme was also shown to generate H₂O₂. Putative nectarin I homologues were found in the nectars of several other plant species.

Floral nectars are often considered as being little more than sugar water. However, closer examination reveals a complex mixture of components. Although simple carbohydrates (*i.e.* sucrose, glucose, and fructose) make up the most significant solutes in nectar, other substances such as amino acids, organic acids, terpenes, flavonoids, glycosides, vitamins, phenolics, oils, and metal ions have also been found in various nectars (1). Enzymatic activities such as invertase, transglucosidase, tyrosinase, phosphatase, oxidase, esterase, and malate dehydrogenase have been suggested to occur in nectars (1). However, these reports have primarily been undetailed investigations, failing to identify the proteins responsible for the respective activities. Only a few investigations have clearly identified the activities of defined nectar proteins (2–5).

* This work was supported by the Carver Trust, the Hatch Act, and State of Iowa funds. This is Journal Paper J-18949 of the Iowa Agriculture and Home Economics Experiment Station (Ames, IA), Project 3340. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, 2212 Molecular Biology Bldg., Iowa State University, Ames, IA 50011. Tel.: 515-294-7885; Fax: 515-294-0453; E-mail: thornr@iastate.edu.

We have previously demonstrated the presence of a limited number of proteins, termed nectarins, that are secreted into the nectar of tobacco flowers (2). The most highly expressed of these proteins, nectarin I, is found only in nectary tissues and to a much lower level in the ovary. Its expression is developmentally regulated, accumulating only at times when nectar is being actively secreted. Following the isolation and characterization of the nectarin I gene, this protein was identified as a germin-like protein (GLP).¹ Germin was first identified in germinating wheat embryos (6). It is a large molecular-weight protein composed of five (6) or six (7, 8) monomer subunits. GLPs have subsequently been identified in all species examined to date from mosses to gymnosperms and dicots to monocots (9–12). Germin is an oxalate oxidase that degrades oxalic acid into H₂O₂ and CO₂ (13–15). Despite the high sequence identity between nectarin I and germin, nectarin I lacks oxalate oxidase activity (2) and consequently has an unknown function. Many other GLPs also lack oxalate oxidase activity (9, 10, 16–19).

Recently, a superoxide dismutase from the moss *Barbula unguiculata*, BuGLP, was isolated and identified as a GLP (9). This fortuitous discovery has led us to examine whether the germin-like protein, nectarin I, is also a superoxide dismutase.

EXPERIMENTAL PROCEDURES

Materials

The plants used for the production of nectarin I have been described previously (2). Additional species examined for the presence of nectarin I are presented in Table I. These plants were obtained from greenhouses on the Iowa State University campus. The species were confirmed at the Iowa State University Herbarium.

Several different superoxide dismutases including the MnSOD from *Escherichia coli* (20), the FeSOD from *E. coli* (21), and the Cu/ZnSOD from bovine erythrocytes (22) were obtained from Sigma and were used without further purification. All other materials were of the highest purity available and were obtained from either Sigma or Fisher.

Purification of Nectarin I

Nectar was collected as described previously (2). The nectarin I protein was obtained in pure form as follows; 12 ml of fresh nectar collected from approximately 500 flowers from 12–15 plants was divided into 600- μ l aliquots in 1.5-ml microcentrifuge tubes. The 600- μ l aliquots were placed in a 90 °C water bath for 45 min, followed by a 30-min centrifugation at 12,000 \times g. To avoid contamination from the pellet, the top 500 μ l of nectar was removed, and 10 ml of 100% (NH₄)₂SO₄ was added to each 1.5 ml of nectar (87% final concentration of (NH₄)₂SO₄) and incubated for 1 h in 15-ml Corex tubes. Following incubation, the tubes were centrifuged at 10,000 \times g for 15 min. The pellets were resuspended in a minimal volume (150 μ l each) of distilled

¹ The abbreviations used are: GLP, germin-like protein; SOD, superoxide dismutase; PAGE, polyacrylamide gel electrophoresis; PAS, periodic acid Schiff; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MOPS, 4-morpholinepropanesulfonic acid; TE-MED, *N,N,N',N'*-tetramethylethylenediamine.

water or 10 mM sodium phosphate, pH 7.8, and dialyzed against 2 L of 10 mM sodium phosphate, pH 7.8, two times. In early studies, partially purified nectar proteins were produced by ammonium sulfate precipitation of raw nectar.

Metal Ion Analysis

Metal ion analysis was performed by flame ionization atomic absorption spectroscopy at the Metal Analysis Laboratory on the Iowa State University campus. All preparations were performed with nitric acid-washed glassware.

Enzyme Assays

Oxalate Oxidase—The procedure described by Sugiura *et al.* (23) was used for the assay of oxalate oxidase activity in solution, using a commercial preparation of barley oxalate oxidase as a positive control.

Superoxide Dismutase—A colorimetric assay (24) using cytochrome *c* as the detector and xanthine-xanthine oxidase as a superoxide generator was utilized in the characterization of purified nectarin I and in the thermostability studies.

SDS-PAGE, Western Blots, and In-gel Staining

SDS-PAGE was performed according to the methods of Laemmli (25). Western blotting was conducted according to methods of Timmons and Dunbar (26). Anti-nectarin I antibodies were described previously (2). Protein concentration was determined by the method of Lowry *et al.* (27).

Detection of Hydrogen Peroxide in Nectar—Hydrogen peroxide in nectar was evaluated as follows. Fifty microliters of nectar was added to 1.95 ml of distilled water, and then 1 ml of developing solution was added. The developing solution contained 80 μ g of 4-aminopyrine, 13 units of horseradish peroxidase and 0.2 μ l of *N,N*-dimethylaniline in 0.1 M sodium phosphate buffer, pH 5.5. After a 10-min incubation at 37 °C, the absorbance was read at 550 nm.

In-gel Staining for Superoxide Dismutase—Negative staining of in-gel SOD activity was performed with nitro blue tetrazolium according to methods outlined by Flohé and Ötting (24). Following electrophoresis, SDS-containing gels were washed in 100 ml of 10 mM sodium phosphate, pH 7.8 (with or without 50 μ M MnSO₄), three times for 30 min each prior to SOD activity staining.

Positive staining of in-gel SOD activity was performed with 4-chloro-1-naphthol. Following SDS-PAGE, gels were washed (three 20-min washes) in 10 mM MOPS, pH 7.0 (with or without 50 μ M MnSO₄). Staining for H₂O₂ production was performed by incubating the washed gels in a staining solution containing: 20 mM MOPS, pH 7.0, 28 μ M riboflavin, 5 units/ml horseradish peroxidase (Sigma), 500 ng/ml 4-chloro-1-naphthol, 10 mM TEMED, and 60% ethanol. Gels were incubated in staining solution in transparent trays on a light box with gentle shaking. Staining was performed for 16–24 h.

Periodic Acid Schiff (PAS) Staining—PAS staining following SDS-PAGE (28) was used to examine nectarin I glycosylation.

Matrix-assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry

MALDI mass spectrometry was used for determining the molecular mass of the purified nectarin I protein. Protein samples of 1–2 μ l containing approximately 2–4 ng of protein were loaded with 1–2 μ l of freshly prepared sinapinic acid matrix onto a time-of-flight mass analyzer (Lasermat 2000 MALDI; Finnigan, Madison, WI). The collected data were analyzed using data processing software (Lasermat 2000). Bovine serum albumin was used as an internal calibration standard.

RESULTS

To evaluate whether nectarin I might be a superoxide dismutase, we initially examined whether raw nectar contained any superoxide dismutase activity. However, the high concentrations of ascorbate present raw nectar interferes with the SOD assay (24), so nectar proteins were precipitated from raw nectar by ammonium sulfate precipitation, and the SOD assay was performed on the partially purified nectarins. Fig. 1 demonstrates that increasing amounts of partially purified nectar proteins result in decreased superoxide-dependent reduction of cytochrome *c*, confirming that the partially purified nectar proteins do indeed contain superoxide dismutase activity.

Because superoxide dismutase activity was identified with the nectar proteins, we next attempted to determine whether

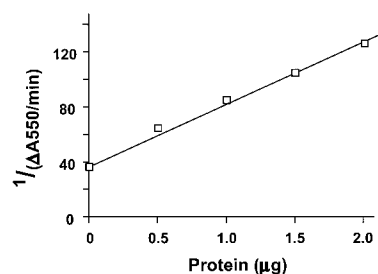


FIG. 1. SOD activity in raw nectar. Nectar proteins purified from raw nectar by ammonium sulfate precipitation were resuspended in 10 mM sodium phosphate, pH 7.8, and dialyzed against the same buffer. SOD activity of crude nectar proteins was then determined by cytochrome *c* reduction according to methods outlined (24). Each point represents the average $1/(\Delta A_{550}/\text{min})$ of three repetitions at 500, 1000, 1500, and 2000 ng of raw nectar proteins.

this superoxide dismutase activity was associated with nectarin I. We ran aliquots of ammonium sulfate-precipitated nectar proteins on native gels and demonstrated that the major nectar protein was stained for superoxide dismutase activity with nitro blue tetrazolium (data not shown).

We have demonstrated previously that even in the presence of SDS the nectarin I protein migrates as an oligomer if the protein samples were not boiled prior to SDS-PAGE (2). We reasoned that if the nonboiled nectarin I protein maintains its oligomeric quaternary structure, perhaps it might also maintain its enzymatic activity. Therefore, we also examined SDS-PAGE gels for superoxide dismutase activity. Fig. 2 (*lane 2*) shows the protein profile of ammonium sulfate precipitated nectar proteins. When the protein samples are prepared in Laemmli buffer without boiling and run on SDS-PAGE gels, the nectarin I migrates as a 165-kDa oligomer. As shown in *lane 3*, Western blotting using antiserum raised against nectarin I identifies the 165-kDa nectarin I oligomer. When a duplicate gel was stained for superoxide dismutase activity (*lane 4*), a band of enzyme activity was observed that corresponded with the 165-kDa nectarin I protein. Thus, nectarin I has superoxide dismutase activity.

We next decided to purify nectarin I and evaluate superoxide dismutase activity on the purified protein. Because GLPs are known for their thermostability (29), and heat precipitation steps are extremely good first steps in the purification of many proteins (30–32), we explored thermostability for the purification of nectarin I from crude nectar proteins. Ammonium sulfate-precipitated nectar proteins were resuspended in 10 mM sodium phosphate buffer, pH 7.8, and dialyzed against this same buffer. Aliquots of these nectar proteins containing 17 μ g of total protein were incubated at various temperatures for 5 min and evaluated for superoxide dismutase activity. As observed in Fig. 3 (*panel A*), the superoxide dismutase activity of nectarin I is remarkably stable over all temperatures up to 90 °C. Above 90 °C, superoxide dismutase activity rapidly declines. We also examined the kinetics of this stability. Aliquots of nectarin I were incubated at temperatures between 80 °C and 95 °C for varying periods of time and immediately placed on ice. The remaining activity of superoxide dismutase was evaluated. As shown in Fig. 3 (*panel B*), nectarin I shows remarkably stable superoxide dismutase activity at temperatures of 90 °C and below. Even for periods as long as 1 h at 90 °C, 85% of superoxide dismutase activity is retained.

When we evaluated the protein profile of the heat-treated, ammonium sulfate precipitated nectar proteins, we were surprised to find that this single thermal denaturation step resulted in the precipitation of all nectar proteins except for nectarin I. This resulted in a two-step, near quantitative purification of nectarin I. We moved the thermal denaturation step

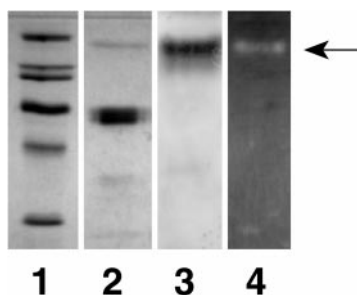


FIG. 2. **In-gel SOD activity.** Nectar proteins purified from raw nectar by ammonium sulfate precipitation were resuspended in 10 mM sodium phosphate, pH 7.8, and dialyzed against the same buffer. These proteins were electrophoresed on a 10% SDS-PAGE. After electrophoresis, the gel was either stained with Coomassie Blue (*lanes 1 and 2*) or processed for in-gel staining of SOD (*lane 4*). The standards used in *lane 1* were: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

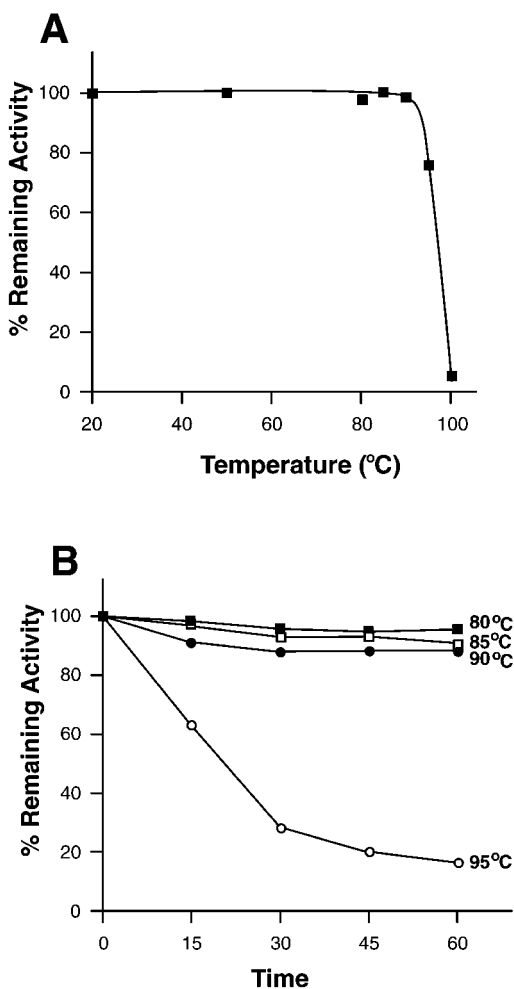


FIG. 3. **Thermostability of nectarin I.** *A*, nectar proteins purified from raw nectar by ammonium sulfate precipitation were resuspended in 10 mM sodium phosphate, pH 7.8, and dialyzed against the same buffer. Aliquots representing approximately 17 μ g of total nectar proteins were incubated at the indicated temperatures for 5 min and cooled on ice. Superoxide dismutase activity was determined by the method of Flohé and Ötting (24). *B*, kinetics of inactivation was determined by evaluating the superoxide dismutase activity following various times of incubation at the indicated temperatures.

prior to the ammonium sulfate precipitation to reduce manipulations. As can be seen in Table I, the recovery of enzyme activity was nearly quantitative. A final specific activity of

2,543 units of superoxide dismutase activity/mg of protein was found for the purified protein. This level of specific activity is similar to that observed with the *E. coli* manganese superoxide dismutase (20).

The purity of the thermostable nectarin I preparation was evaluated by SDS-PAGE. Fig. 4 shows the protein profile of crude nectar in *lanes 1* (nonboiled) and *2* (boiled) and of the purified nectarin I preparation in *lanes 3* (nonboiled) and *4* (boiled). As can be observed, in nonboiled nectar, the nectarin I oligomer migrates at 165 kDa, whereas the monomer migrates at 29 kDa (compare *lanes 1 and 2*). The purified nectarin I preparation also gives a single 165-kDa band on the gel when nonboiled (*lane 3*) and a single 29-kDa band following boiling (*lane 4*). Based upon these observations, we concluded that nectarin I was pure.

This figure also demonstrates that the oligomeric form of nectarin I binds Coomassie Blue much less effectively than the monomeric form. Each pair of these lanes, 2 and 3, or 4 and 5, contains the same amount of protein, but clearly the monomeric form gives greater interaction with the Coomassie Blue stain.

The SDS-PAGE analysis of nectarin I shows a molecular mass of 29 kDa. However, the MALDI-TOF analysis of purified nectarin I showed a M^+ peak of $22,533 \pm 58$ ($n = 5$). The M_2^+ peak was also readily detected with a mass of $45,184 \pm 131$ ($n = 5$). Larger complexes are not observed. This discrepancy in molecular masses between the SDS-PAGE and MALDI likely results from the extreme stability of the nectarin I protein during electrophoresis. If the protein is not completely unfolded and coated with SDS, then the protein would be expected to run slower than expected, producing an artificially high molecular mass on the SDS-PAGE.

The molecular mass of the mature nectarin I protein predicted from the amino acid sequence is 21,062 Da (2). The difference between the predicted molecular mass and that found by mass spectrometry, 1,471 Da, is unaccounted for. However, it is known that GLPs are glycosylated (33). All GLPs, including nectarin I, contain a conserved site of *N*-glycosylation (2, 10, 11, 16, 17, 19, 33). PAS staining (28) demonstrated the presence of carbohydrate on the purified nectarin I protein (Fig. 4, *lanes 6 and 7*). Jaikaran *et al.* (33) have reported the structure of the *N*-linked glycan from wheat germin. That structure is a biantennary nonasaccharide with the composition $(\text{GlcNAc})_4:\text{Man}_3:\text{Xyl}:\text{Fuc}$. This nonasaccharide has a molecular mass of 1,576 Da, which corresponds well with the mass differences observed between the MALDI-TOF analysis and the cDNA-predicted molecular mass (1,471 Da). Therefore, we expect that the nectarin I glycan is highly similar to the *N*-linked glycan present on wheat germin.

To determine whether the purified nectarin I had superoxide dismutase activity, we next evaluated the ability of the purified nectarin I to remove superoxide generated by xanthine-xanthine oxidase. As can be seen in Fig. 5, the purified protein was indeed able to dismutate superoxide in a dose-dependent manner. Therefore, we conclude that the superoxide dismutase activity associated with tobacco nectar is due to the presence of nectarin I.

Based upon the type of metals that they contain, there are three known families of superoxide dismutases: FeSOD, Cu/ZnSOD, and MnSOD. To determine the type of superoxide dismutase family to which nectarin I belongs, we analyzed the purified nectarin I protein for metal ions. This analysis demonstrated the presence of 0.5 mol of manganese/mol of nectarin I monomer. Iron and copper were present in trace amounts at or near the limits of detection.

To confirm that nectarin I was a manganese superoxide

TABLE I
Purification of nectarin I

Nectar was harvested as described under "Experimental Procedures."

	SOD activity ^a	Protein ^b	Recovery		Specific activity	Purification
			Activity	Protein		
	units	mg		%	units/mg protein	-fold
Raw nectar ^c	1,048	1.35	100	100	845	1.00
Heat treatment	1,048	0.79	100	63.7	1377	1.57
(NH ₄) ₂ SO ₄ precipitation	966	0.38	92.2	30.6	2543	3.01

^a Superoxide dismutase activity was evaluated by the method of Flohé and Ötting (24). One unit of activity is defined as the amount of enzyme resulting in 50% inhibition of cytochrome *c* reduction under standard conditions.

^b Protein was evaluated by the method of Lowry (27).

^c To quantitate recovery for this purification, raw nectar was initially dialyzed *versus* 10 mM sodium phosphate buffer, pH 7.8, to remove materials that interfere with the Lowry protein assay. This dialysis is not required for the purification of nectarin I.

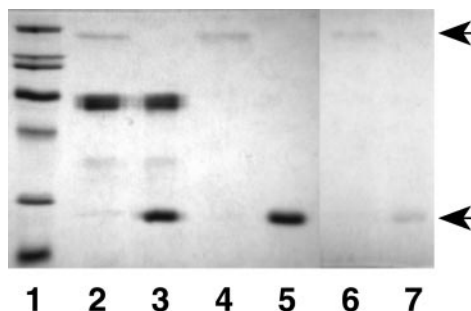


FIG. 4. Purification of nectarin I. Nectarin I was purified from nectar by heat precipitation of the other nectar proteins as described under "Experimental Procedures." Crude nectar, nonboiled and boiled (lanes 2 and 3, respectively), and 20 μ g of pure nectarin I, nonboiled and boiled (lanes 4 and 6 and lanes 5 and 7, respectively) were subjected to 12% SDS-PAGE and stained with Coomassie Blue (lanes 4 and 5) or by PAS (lanes 6 and 7). The standards used in lane 1 were: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

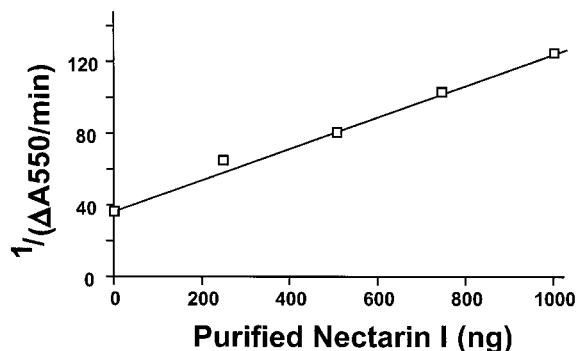


FIG. 5. SOD activity of purified nectarin I. Nectarin I was purified from nectar as described under "Experimental Procedures." SOD activity of purified nectarin I was then determined by cytochrome *c* reduction according to methods outlined (24). Each point represents the average $1/(\Delta A_{550}/\text{min})$ of three repetitions at 250, 500, 750, and 1000 ng of purified nectarin I.

dismutase, hydrogen peroxide inhibition studies of enzyme activity were performed. Manganese superoxide dismutases are stable in the presence of 5 mM H₂O₂, whereas copper/zinc and iron superoxide dismutases lose activity following this treatment (34, 35). Because nectarin I retains superoxide dismutase activity following SDS-PAGE (see Fig. 2, lane 3), we examined this inhibition following gel electrophoresis. Lanes 1 and 3 of Fig. 6 contain a mixture of commercially available superoxide dismutases, including the manganese superoxide dismutase from *E. coli* (20), the iron superoxide dismutase from *E. coli* (21), and the copper/zinc superoxide dismutase from bovine erythrocytes (22). Lanes 2 and 4 contain purified nectarin I.

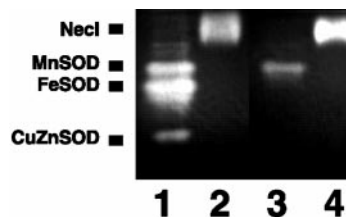


FIG. 6. Nectarin I SOD activity is insensitive to treatment with H₂O₂. Lanes 1 and 3 contain 30 units each of the MnSOD from *E. coli* (20), the FeSOD from *E. coli* (21), and the Cu/ZnSOD from bovine erythrocytes (22). Lanes 2 and 4 contain 25 μ g of purified nectarin I. The proteins were electrophoresed on duplicate 10% SDS-polyacrylamide gels. Following electrophoresis, the gels were bathed for 1 h in either 10 mM sodium phosphate, pH 7.8 (lanes 1 and 2), or 10 mM sodium phosphate, pH 7.8, containing 5 mM H₂O₂. Following incubation, the gels were stained for superoxide dismutase activity (24).

The nonboiled proteins were all separated on SDS-PAGE gels and stained for superoxide dismutase activity with nitroblue tetrazolium (24). As can be observed in lanes 1 and 2, each of the nonboiled proteins retains superoxide dismutase activity following SDS-PAGE. Treatment of these proteins for 1 h with 5 mM H₂O₂, however, results in the loss of activity of the iron and copper/zinc superoxide dismutases. In contrast, both the manganese superoxide dismutase from *E. coli* and the nectarin I superoxide dismutase remain active following this treatment. Similarly, NaCN is capable of inactivating Cu/Zn superoxide dismutases (34, 35). Incubation with NaCN did not inhibit the enzymatic activity of nectarin I (data not shown).

Although nonboiled nectarin I retains its quaternary structure and its superoxide dismutase activity during SDS-PAGE, it decomposes to its monomeric form following boiling. This monomeric form does not retain the superoxide dismutase activity after boiling (compare lanes 1 and 2 of Fig. 7). We therefore decided to test whether we could reactivate the superoxide dismutase activity following metal ion replacement. As shown in Fig. 7, the addition of 50 mM FeSO₄, CuSO₄, ZnCl₂, or CuSO₄/ZnCl₂ to the gel wash solutions failed to reactivate the superoxide dismutase activity (lanes 3, 4, 5, or 6). In contrast, addition of 50 mM MnSO₄ produced active enzyme (lane 7). Thus, only manganese was able to reconstitute enzyme activity, confirming that the nectarin I is a manganese superoxide dismutase.

The studies illustrated in Fig. 7 do not provide information about the quaternary structure of the active form of nectarin I following metal ion replacement. To examine this in more detail, we first inactivated the superoxide dismutase activity by boiling. After separating the monomeric form on an SDS-PAGE gel, we renatured the enzyme as in lane 7 of Fig. 7. The renaturation was verified by staining the gel for superoxide dismutase activity (data not shown). Subsequently, duplicate slices of the active protein were excised from the gel and re-

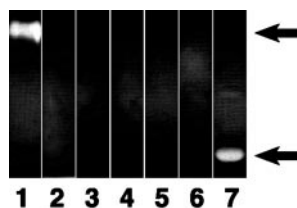


FIG. 7. Manganese ions restore SOD activity. For each lane, 20 μg of purified nectarin I was run on a 10% SDS-PAGE following boiling for 3 min in Laemmli buffer (25) lacking β -mercaptoethanol. Lane 1 was not boiled. Subsequently, each gel was washed (three 30-min washes) in 10 mM sodium phosphate buffer, pH 7.8, containing either no metals (lane 2) or 50 μM FeSO_4 (lane 3), CuSO_4 (lane 4), ZnCl_2 (lane 5), both CuSO_4 and ZnCl_2 (lane 6), or MnSO_4 (lane 7). Following incubation, the gels were stained for superoxide dismutase activity (24).

electrophoresed on a second SDS-PAGE.

Fig. 8 (panel A) shows the Coomassie staining of the reassociated protein. Clearly the majority of the enzyme is still present in the monomeric form. However, a significant amount of the protein has reassociated into dimer, trimer, tetramer, and pentamer forms. This ladder of assembly is best observed if β -mercaptoethanol is included in the original boiling step (lane 2). If the β -mercaptoethanol is not included in the original boiling step, the intermediate forms are reduced but the pentameric form is present in higher amounts. Inclusion of β -mercaptoethanol results in increased accumulation of the dimeric and trimeric forms of the reassociated protein. When we examine the enzymatic activity of these forms (lanes 4 and 5), we observe that only the tetrameric and pentameric forms have enzymatic activity. Thus, the reconstitution of the superoxide dismutase activity of the nectarin I enzyme requires the re-multimerization of the nectarin I monomers.

Interestingly, when the nectarin I monomers are incubated in the absence of manganese, a small portion of the monomeric form reassociates to form a multimeric form (lane 3); however, without manganese, this reassociated form is not active (lane 6).

Superoxide dismutase converts O_2^- into H_2O_2 . The superoxide dismutase assay used in all of the above in-gel studies (24) monitors the clearance of riboflavin-generated superoxide. To further confirm the superoxide dismutase activity of nectarin I, we also examined whether the manganese-reactivated enzyme was capable of generating H_2O_2 .

Fig. 9 shows the results of these studies. In panel A, we monitored the dismutation of flavin-generated superoxide by the native 165-kDa form of nectarin I (lanes 1 and 3) and of the manganese-reactivated form (lane 4). In panel B, we used a 4-chloro-1-naphthol stain to show the direct generation of H_2O_2 . The pattern of hydrogen peroxide staining in panel B correlates exactly with loss of superoxide in panel A, confirming both the loss of the substrate, superoxide, and the generation of the product, H_2O_2 , for both the native and the manganese-reactivated enzymes.

Because the dismutation of superoxide results in the generation of H_2O_2 , we examined whether plant nectar contains H_2O_2 . We examined nectar from mature, opened flowers from a series of 10 greenhouse-grown plants. These plants showed a range of H_2O_2 accumulation from $<20 \mu\text{M}$ to $>4000 \mu\text{M}$ with a mean value of $771 \mu\text{M}$. This is significantly higher than the levels of H_2O_2 (10–100 μM) that are normally toxic to cells (36). SDS-PAGE analysis demonstrated that nectarin I was present in the nectars of each of these 10 plants.

Finally, to investigate whether nectarin I proteins are found in other plant species, we visited all greenhouses present on the Iowa State University campus and obtained nectar from all plants that produced nectar in sufficient quantities for analy-

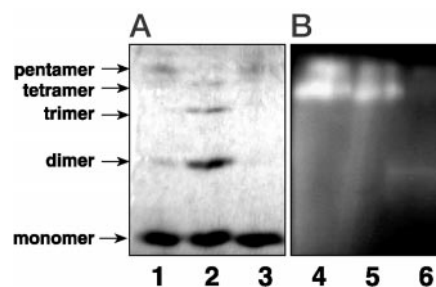


FIG. 8. Quaternary structure of nectarin I following Mn^{2+} re- placement. Twenty-five micrograms of purified nectarin I was boiled in Laemmli buffer with or without β -mercaptoethanol and subjected to 10% SDS-PAGE. Duplicate gels were then washed in 10 mM NaPO_4 (three 30-min washes) either with or without 50 μM MnSO_4 followed by SOD activity staining (24). The duplicate activity/protein bands were excised from the gels and incubated in $1\times$ Laemmli buffer for 20 min and again subjected to 10% SDS-PAGE (3 mm thick) and either stained for SOD activity (panel B) or stained with Coomassie Blue R-250 (panel A). Lanes 1 and 4 contain samples that were boiled without β -mercaptoethanol (prior to the first SDS-PAGE) and were reconstituted with 50 μM MnSO_4 (prior to activity staining of the first SDS-PAGE). Lanes 2 and 5 contain samples that were boiled with β -mercaptoethanol and were reconstituted with 50 μM MnSO_4 . Lanes 3 and 6 contain samples that were boiled without β -mercaptoethanol and were not reconstituted with 50 μM MnSO_4 .

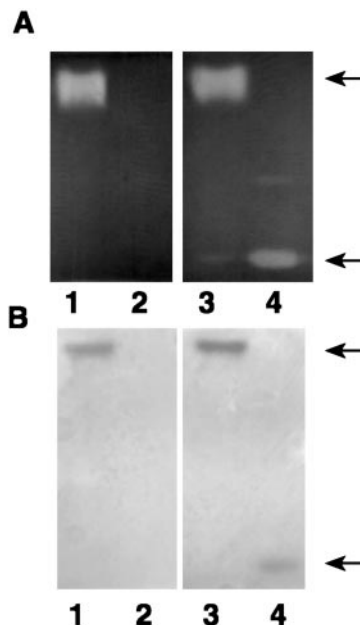


FIG. 9. Manganese-reconstituted nectarin I generates hydro- gen peroxide. Panel A, 25 μg of purified nectarin I was electrophoresed on duplicate 10% SDS-PAGE either without boiling (lanes 1 and 3) or following boiling for 3 min in Laemmli buffer (25) lacking β -mercaptoethanol (lanes 2 and 4). Following electrophoresis the gels were washed in 10 mM sodium phosphate buffer, pH 7.8, containing either no metals (lanes 1 and 2) or 50 μM MnSO_4 (lanes 3 and 4) and stained for superoxide dismutase activity (24). Panel B, the gels were prepared in an identical manner as in panel A. Following electrophoresis, the gels were washed (three 20-min washes) in 100 ml of 10 mM MOPS, pH 7.0, either without metals (lanes 1 and 2) or containing 50 μM MnSO_4 (lanes 3 and 4). The gels were then stained for nectarin I-generated H_2O_2 as follows. After washing in MnSO_4 -containing buffer as above, the gel was briefly washed (15 min) in buffer without any added metals, and then a staining solution, containing 20 mM MOPS, pH 7.0, 28 μM riboflavin, 5 units/ml horseradish peroxidase, 0.5 mg/ml 4-chloro-1-naphthol, 10 mM TEMED, and 60% ethanol was added. The gels were incubated in this staining solution in transparent trays on a light box with gentle shaking. Staining continued for 16–24 h.

sis. These nectar samples were electrophoresed on SDS-PAGE gels, and nectarin I immuno-cross-reactive material was visualized by Western blot analysis using antibodies raised against

TABLE II
Phylogenetic identification of nectarin I-like proteins

Species ^a	Common name ^b	Family	Nectarin I-positive
<i>Abutilon hybridum</i>	^c	Malvaceae	–
<i>Burgmansia xcadida</i>	Angel's trumpet	Solanaceae	+
<i>Camellia japonica</i>	Yabu-Tubaki	Theaceae	+
<i>Columnnea longifolia</i>	Woundwort	Gesneriaceae	–
<i>Ixora chinensis</i>	Pechar periok (broken pot)	Rubiaceae	–
<i>Kohleria bogotensis</i>	^c	Gesneriaceae	–
<i>Nepenthes superba</i> ^d	Monkey slipper	Nepenthaceae	+
<i>Nicotiana glauca</i>	Flowering tobacco	Solanaceae	+
<i>Nicotiana plumbaginifolia</i>	Wild tobacco	Solanaceae	+
<i>Nicotiana tabacum</i>	Common tobacco	Solanaceae	+
<i>Philodendron domesticum</i>	Philodendron	Araceae	+
<i>Rhoeo spathae</i>	Barquito	Commelinaceae	–
<i>Sarracenia purpurea</i> ^d	Saddleplant	Sarracenaceae	+
<i>Strelitzia reginae</i>	Bird of paradise	Strelitziaceae	+
<i>Thunbergia affinis</i>	Akar	Acanthaceae	–

^a Species names were confirmed by the Iowa State University Herbarium.

^b Common names were obtained from the EthnoBot data base available from the United States Department of Agriculture web site.

^c Common name was not available.

^d The nectar from the pitcher plants was nonfloral nectar.

nectarin I (2). In this analysis we examined 15 species from 11 different plant families (Table II). Six different plant families (Araceae, Nepenthaceae, Sarraceniaceae, Solanaceae, Strelitziaceae, and Theaceae) showed nectarin I-cross-reactive proteins. These cross-reactive proteins were either 29-kDa nectarin I-like proteins or were >150 kDa. The >150-kDa proteins were observed in at least three different species, *Sarracenia purpurea*, *Nepenthes superba*, and *Strelitzia reginae*.

DISCUSSION

Nectarin I is a germin-like protein that has manganese superoxide dismutase activity. This enzymatic activity is remarkably thermostable, maintaining high activity even when incubated at 90 °C for 1 h. This thermostability allowed for a facile purification of the nectarin I protein. The purified nectarin I protein contained manganese, and the superoxide dismutase activity was resistant to inhibition by both H₂O₂ and NaCN, compounds that inhibit the activity of iron and copper/zinc superoxide dismutases but not manganese superoxide dismutases. Following disassociation of the nectarin I protein into monomers, the enzymatic activity could be reconstituted upon the addition of manganese, but not with iron, copper, zinc, or copper/zinc. Taken together, these data indicate that nectarin I is a manganese superoxide dismutase.

Further, this manganese superoxide dismutase is uncommonly stable. Not only is it highly resistant to thermal denaturation, but it also maintains both its quaternary structure and enzymatic activity when electrophoresed in the presence of SDS. Only by boiling were we able to disassociate nectarin I into its monomeric components.

Following removal of SDS and addition of manganese, these monomers readily reassociated into oligomeric forms of the enzyme. The predominant forms of the reassociated enzyme could be influenced by the disassembly procedure. If the disassembly of the enzyme was performed by boiling in the absence of β -mercaptoethanol, the predominant form of the reassociated enzyme was the pentamer. However, if disassembly was performed by boiling in the presence of β -mercaptoethanol, dimer and trimers were the principal reassociated forms. These smaller forms lacked enzyme activity. Only the tetrameric and pentameric forms of the reassociated enzyme showed superoxide dismutase activity. The reason why addition of β -mercaptoethanol in the disassembly process results in the smaller reassociated forms is not clear; however, the nectarin I protein

does contain a pair of cysteine residues at positions 10 and 25 of the mature protein. Apparently, reduction of this disulfide pair results in a nectarin I that inhibits the formation of the higher multimeric forms.

Based upon conservation of sequence identity among a large number of GLPs, the location of the metal binding site has been proposed to consist of a cluster of three histidine residues, numbered His-85, His-87, and His-131, in the mature nectarin I protein. Two models for oxalate oxidase have been published, based upon the structure of vicilin (7), and on the C-terminal domain of jack bean canavalin (37). Both of these models predict that these three histidines lie on neighboring anti-parallel β -strands, and that the side chains form a cluster that is reminiscent of other metal-binding sites. Both models also predict that the side chain of a glutamate residue lies close to the histidine cluster and may function as a fourth ligand of the manganese. This glutamate is also conserved in the mature nectarin I protein as Glu-92. Wheat germin and nectarin I share 51.7% identity, rising to 60.7% if conservative substitutions are permitted. Although nectarin I is clearly a germin-like protein, despite repeated efforts, we have found that nectarin I does not have oxalate oxidase activity. Similar observations have been made for a number of other GLPs (9, 10, 16–19). We have also tested wheat oxalate oxidase for superoxide dismutase activity and have found none. Therefore, if the conserved histidines and glutamate are involved in the oxalate oxidase activity of the wheat germin, then other factors that are missing in nectarin I must also participate to result in the oxidation of oxalic acid and likewise in the dismutation of superoxide.

The GenBank[®] contains at least 70 full-length or near full-length sequences encoding germin-like proteins. Our analysis of 73 GLP sequences (see Fig. 10) has identified five phylogenetic clades (38). The true germin clade contains most of the wheat and barley germains along with one *Arabidopsis*, one rice, and one maize GLP (total of 14 sequences). We also identify a small clade of three sequences, referred to as the gymnosperm GLPs. This clade contains the *Pinus sp.* GLPs and one *Arabidopsis* GLP (GLP7). With the exception of a single outlier, all of the remaining plant GLPs fall into three families: subfamily 1 (31 members), subfamily 2 (11 members), and subfamily 3 (13 members).

The wheat and barley germains (true germin clade) have

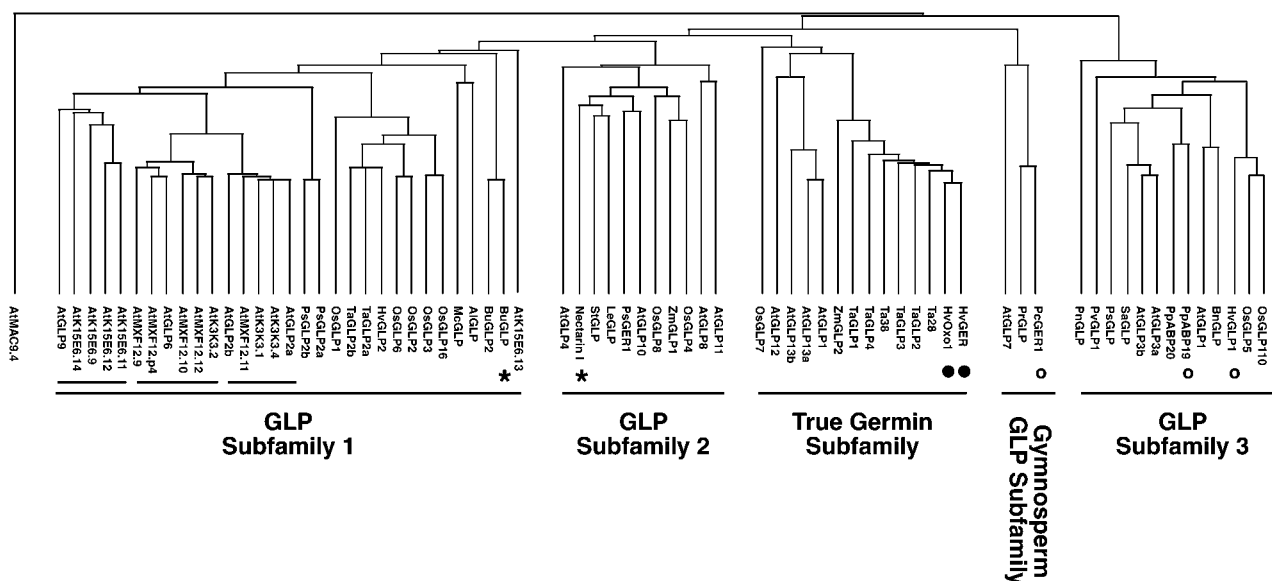


FIG. 10. Phylogenetic analysis of 73 germin-like protein sequences. Those proteins that have been analyzed for enzymatic activity are marked. An asterisk indicates that the protein has superoxide dismutase activity and no oxalate oxidase activity. Open circles indicate that the protein has no oxalate oxidase activity but has not been assayed for superoxide dismutase activity. Closed circles indicate that the protein has oxalate oxidase activity. The GenBank[®] accession numbers of the sequences analyzed are: *OsGLP10*, AF051156; *OsGLP5*, AF032975; *HuGLP1*, Y15962; *BnGLP*, U21743; *AtGLP1*, U75206; *PpABP19*, U79114; *PpABP20*, U81162; *AtGLP3a*, U75188; *AtGLP3b*, U75195; *SaGLP*, X84786; *PsGLP*, AJ222979; *PvGLP1*, AJ276491; *PnGLP*, D45425; *PcGER1*, AF039201; *PrGLP*, AF049065; *AtGLP7*, AF170550; *HuGER*, L15737; *HuOx1*, P45851; *Ta28*, M63223; *TaGLP2*, Y09916; *TaGLP3*, Y09917; *Ta38*, M63224; *TaGLP4*, Y09918; *TaGLP1*, Y09915; *ZmGLP2*, AF261942; *AtGLP1*, U75206; *AtGLP13a*, AAF79304; *AtGLP13b*, AAF79303; *AtGLP12*, AAD55294; *OsGLP7*, AF072694; *AtGLP11*, AF058914; *AtGLP8*, U75207; *OsGLP4*, AF032974; *ZmGLP1*, AF261941; *OsGLP8*, AF072695; *AtGLP10*, U95036; *PsGER1*, AJ250832; *LeGLP*, AB012138; *StGLP*, AF067731; *Nectarin I*, AF132671; *AtGLP4*, U75187; *AtK15E6.13*, AB009048; *BuGLP*, AB028460; *BuGLP2*, AB028454; *AiGLP*, AB024338; *McGLP*, M93041; *OsGLP16*, AF042489; *OsGLP3*, AF032973; *OsGLP2*, AF141879; *OsGLP6*, AF032976; *HuGLP2*, X93171; *TaGLP2a*, AJ237942; *TaGLP2b*, AJ237943; *OsGLP1*, AF141880; *PsGLP2a*, AJ250833; *PsGLP2b*, AJ250834; *AtGLP2a*, U75192; *AtK3K3.4*, AB010694; *AtK3K3.1*, AB010694; *AtMXF12.11*, AB016892; *AtGLP2b*, X91957; *AtK3K3.2*, AB010694; *AtMXF12.12*, AB016892; *AtMXF12.10*, AB016892; *AtGLP6*, U75194; *AtMXF12p4*, AB016892; *AtMXF12.9*, AB016892; *AtK15E6.11*, AB009048; *AtK15E6.12*, AB009048; *AtK15E6.9*, AB009048; *AtK15E6.14*, AB009048; *AtGLP9*, U81294; *AtMAC9.4*, AB010069.

oxalate oxidase activity. Outside of the true germin clade, oxalate oxidase activity has not been reported, although the number of proteins tested is still quite small. The moss GLP (9) that is a superoxide dismutase and has no detectable oxalate oxidase activity is a member of subfamily 1. Nectarin I, which also has superoxide dismutase activity and no detectable oxalate oxidase activity, is a member of subfamily 2. The peach auxin-binding protein and a barley GLP, both belonging to subfamily 3, have been tested for oxalate oxidase activity, and none could be detected (17, 19). Likewise, no oxalate oxidase activity could be detected for the *Pinus caribaea* GLP (gymnosperm GLP clade) (16). These three proteins have not been tested for superoxide dismutase activity.

Based upon this limited analysis, it appears that oxalate oxidase activity is associated with only one group of GLPs representing only 15% (11/70) of all GLPs. It is too soon to tell whether superoxide dismutase activity is common among the GLPs. Nevertheless, the finding that members of two separate clades (subfamilies 1 and 2) of the phylogenetic tree contain GLPs with superoxide dismutase activity implies that superoxide dismutase activity may be widespread throughout this protein family.

The biochemical role of germin-like proteins in plants has received much attention. Numerous functions have been proposed for GLPs, including desiccation and hydration (39), restructuring of cell walls (13), salt and heavy metal response (40), and plant defenses (15, 41, 42). Because GLPs represent a large family of extracellular proteins, some of which have superoxide dismutase activity, we propose a novel function for these proteins in mediating the oxidative burst during the wound response.

When plants are wounded, they respond by activating a large number of genes that function to close and seal the wound site,

alter hormonal homeostasis, inhibit photosynthetic translation, and to activate proteinaceous and phytoalexin defense responses (43). Very early in this response, NADPH oxidase is activated, which releases O_2^- into the extracellular compartment (44). The dismutation of O_2^- into H_2O_2 is a necessary intermediate step in this process because the oxidative burst is completely inhibited by catalase (45, 46). Despite the importance of superoxide dismutase activity in this pathway, enzymes catalyzing this step have not yet been identified. Because of the extracellular localization and the near ubiquitous distribution throughout plant tissues, GLPs could provide the superoxide dismutase activity required for the production of H_2O_2 .

In addition to the functional role of GLPs throughout the plant, we propose that nectarin I has an additional role in nectar, to protect the reproductive tissues from microbial attack. Nectar is offered by plants to insect and avian pollinators to increase the efficiency of seed set. Nectar is secreted from the nectary, a ring of cells surrounding the base of the ovary, and bathes the gynoecium. Indiscriminate floral visitation by pollinators must certainly transfer microorganisms. Although the rich milieu of nectar nutrients would make an ideal growth medium for microbes, microbial colonization of the fluid bathing the ovary would be evolutionarily unfavorable. We propose that the high level of H_2O_2 in nectar functions to protect the reproductive tissues from microbial infection. H_2O_2 is generally toxic to cells at relatively low levels; on the order of 10 to 100 μM (36). The levels that we have observed in the nectar of tobacco plants are significantly higher than this (<20 μM to >4000 μM).

That nectarin I-immunoreactive proteins were identified in the nectars of a number of other plant families indicates that this method of protection may be widespread within the plant

kingdom. The high levels of H₂O₂ found to be present in nectar also correlates with the finding that peroxidase and catalase activity are abundant in the gut and malpighian tubules of insects (47–50).

Acknowledgment—We thank Wade Johnson for assistance with the MALDI-TOF analysis.

REFERENCES

- Baker, H. G., and Baker, I. (1983) in *The Biology of Nectaries* (Bentley, B., and Elias, T., eds.) pp. 126–152, Columbia University Press, New York
- Carter, C., Graham, R., and Thornburg, R. W. (1999) *Plant Mol. Biol.* **41**, 207–216
- Peumans, W. J., Smeets, K., Van Nerum, K., Van Leuven, F., and Van Damme, E. J. M. (1997) *Planta* **201**, 298–302
- Scala, J., Iott, K., Schwab, W., and Semersky, F. E. (1969) *Plant Physiol.* **44**, 367–371
- Heslop-Harrison, Y., and Knox, R. B. (1971) *Planta* **96**, 183–211
- McCubbin, W. C., Cyril, M. K., Kennedy, T. D., and Lane, B. G. (1987) *Biochem. Cell Biol.* **65**, 1039–1048
- Gane, P. J., Dunwell, J. M., and Warwicker, J. (1998) *J. Mol. Evol.* **46**, 488–493
- Woo, E.-J., Dunwell, J. M., Goodenough, P. W., and Pickersgill, R. W. (1998) *FEBS Lett.* **437**, 87–90
- Yamahara, T., Shiono, Y., Suzuki, T., Tanaka, K., Takio, S., Sato, K., Yamazaki, S., and Satoh, T. (1999) *J. Biol. Chem.* **274**, 33274–33278
- Domon, J.-M., Dumas, B., Lainé, E., Meyer, Y., Alain, D., and David, H. (1995) *Plant Physiol.* **108**, 141–148
- Carter, C., Graham, R., and Thornburg, R. W. (1998) *Plant Mol. Biol.* **38**, 929–943
- Rahman, S., Grzelczak, Z., Kennedy, T., and Lane, B. (1988) *Biochem. Cell Biol. Biochim. Biol. Cell* **66**, 100–106
- Lane, B. G., Dunwell, J. M., Ray, J. A., Schmitt, M. R., and Cuming, A. C. (1993) *J. Biol. Chem.* **268**, 12239–12242
- Dumas, B., Sailland, A., Cheviet, J. P., Freyssinet, G., and Pallett, K. (1993) *C. R. Acad. Sci. III* **316**, 793–798
- Zhang, Z., Collinge, D. B., and Thordal-Christensen, H. (1995) *Plant J.* **8**, 139–145
- Neutelings, G., Domon, J., Membre, N., Bernier, F., Meyer, Y., David, A., and David, H. (1998) *Plant Mol. Biol.* **38**, 1179–1190
- Vallelain-Bindschedler, L., Mösinger, E., Métraux, J.-P., and Schweizer, P. (1998) *Plant Mol. Biol.* **37**, 297–308
- Wei, Y., Ziguio, Z., Andersen, C. H., Schmelzer, E., Gregersen, P. L., Collinge, D. B., Smedegaard-Petersen, V., and Thordal-Christensen, H. (1998) *Plant Mol. Biol.* **36**, 101–112
- Ohmiya, A., Tanaka, Y., Kadowaki, K., and Hayashi, T. (1998) *Plant Cell Physiol.* **39**, 492–499
- Keele, B., Jr., McCord, J., and Fridovich, I. (1970) *J. Biol. Chem.* **245**, 6176–6181
- Yost, F. J., and Fridovich, I. (1973) *J. Biol. Chem.* **248**, 4905–4908
- Steinman, H., Naik, V., Abernethy, J., and Hill, R. (1974) *J. Biol. Chem.* **249**, 7326–7338
- Sugiura, M., Yamamura, H., Haramo, K., Sasaki, M., Morikava, M., and Tsuboi, M. (1979) *Chem. Pharm. Bull.* **27**, 2003–2007
- Flohé, L., and Ötting, F. (1984) *Methods Enzymol.* **105**, 93–104
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Timmons, E. D., and Dunbar, B. S. (1990) *Methods Enzymol.* **182**, 679–687
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Zacharius, R. M., Zell, T. E., Morrison, J. M., and Woodlock, J. J. (1969) *Anal. Biochem.* **30**, 148–152
- Lane, B. G., Cuming, A. C., Frégeau, J., Carpita, N. C., Hurkman, W. J., Bernier, F., Dratewka-Kos, E., and Kennedy, T. D. (1992) *Eur. J. Biochem.* **209**, 961–969
- Bryant, J., Green, T. R., Gurusaddaiah, T., and Ryan, C. A. (1976) *Biochemistry* **15**, 3418–3424
- Sommert, U., Traving, C., and Schauer, R. (1999) *Glycoconj. J.* **16**, 425–435
- McManaman, J., Shellman, V., Wright, R., and Repine, J. (1996) *Arch. Biochem. Biophys.* **332**, 135–141
- Jaikaran, A. S. I., Kennedy, T. D., Dratewka-Kos, E., and Lane, B. G. (1990) *J. Biol. Chem.* **265**, 12503–12512
- Koster, J. F., Slee, R. G., and Van Berkel, T. J. C. (1980) in *Biological and Clinical Aspects of Superoxide and Superoxide Dismutase* (Bannister, W. H., and Bannister, J. V., eds) Elsevier, New York
- Geller, B. L., and Winge, D. R. (1983) *Anal. Biochem.* **128**, 86–92
- Halliwell, B., and Gutteridge, J. M. C. (1999) *Free Radicals in Biology and Medicine*, Oxford University Press, New York
- Requena, L., and Bornemann, S. (1999) *Biochem. J.* **343**, 185–190
- Carter, C., and Thornburg, R. W. (1999) *J. Plant Biol.* **42**, 97–108
- Lane, B. G. (1991) *FASEB J.* **5**, 2893–2901
- Hurkman, W. J., Tao, H. P., and Tanaka, C. K. (1991) *Plant Physiol.* **97**, 366–374
- Hurkman, W. J., and Tanaka, C. K. (1996) *Plant Physiol.* **111**, 735–739
- Dumas, B., Freyssinet, G., and Pallett, K. (1995) *Plant Physiol.* **107**, 1091–1096
- Zhou, L., and Thornburg, R. W. (1999) in *Inducible Gene Expression in Plants* (Reynolds, P., ed.) pp. 127–167, CAB International, Wallingford, Oxon, United Kingdom
- Wojtaszek, P. (1997) *Biochem. J.* **322**, 681–692
- Lindner, W. A., Hoffman, C., and Grisebach, H. (1988) *Phytochemistry* **27**, 2501–2503
- Collén, J., and Pderesén, M. (1994) *Physiol. Plant.* **92**, 417–422
- Felton, G., and Summers, C. (1995) *Arch. Insect Biochem. Physiol.* **29**, 187–197
- Beard, M., and Holtzman, E. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7433–7437
- de Azeredo-Oliveira, M., and Mello, M. (1998) *Cytobios* **93**, 83–92
- Ahmad, S., Pritsos, C., Bowen, S., Heisler, C., Blomquist, G., and Pardini, R. (1988) *Free Radical Res. Commun.* **4**, 403–408