Subunit structure of vacuolar proton-pyrophosphatase as determined by radiation inactivation

Chi M. TZENG, Chih Y. YANG, Su J. YANG, Shih S. JIANG, Soong Y. KUO, Shu-Hsien HUNG, Jing T. MA and Rong L. PAN*

Institute of Radiation Biology and Department of Nuclear Science, College of Nuclear Science, National Tsing Hua University, Hsin Chu, 30043 Taiwan, Republic of China

Vacuolar proton-pyrophosphatase (H^+ -PPase) of mung bean seedlings contains a single kind of polypeptide with a molecular mass of approx. 73 kDa. However, in this study, a molecular mass of approx. 140 kDa was obtained for the purified vacuolar H^+ -PPase by size-exclusion gel-filtration chromatography, suggesting that the solubilized form of this enzyme is a dimer. Radiation inactivation analysis of tonoplast vesicles yielded functional masses of 141.5 ± 10.8 and 158.4 ± 19.5 kDa for PP_i hydrolysis activity and its supported proton translocation respectively. These results confirmed the *in situ* dimeric structure of the membrane-bound H^+ -PPase of plant vacuoles. Further target-size analysis showed that the functional unit of purified vacuolar

H^+ -PPase was 71.1 ± 6.7 kDa, indicating that only one subunit of the purified dimeric complex would sufficiently display its enzymic reaction. Moreover, in the presence of valinomycin and KCl, the functional size of membrane-bound H^+ -PPase was decreased to approx. 63.4 ± 6.3 kDa. A working model was proposed to elucidate the structure of native H^+ -PPase on vacuolar membrane as a functional dimer. Factors that would disturb the membrane, e.g. membrane solubilization and the addition of valinomycin and KCl, may induce an alteration in its enzyme structure, subsequently resulting in a different functional size.

INTRODUCTION

Tonoplast of higher plants contains two different, novel types of proton-pumping enzymes, H^+ -translocating ATPase (EC 3.6.1.3) and H^+ -pyrophosphatase (H^+ -PPase, EC 3.6.1.1) for a common function [1–3]. By using different substrates, both vacuolar enzymes catalyse electrogenic proton translocation to generate a similar inside-acid, inside-positive electrochemical potential for secondary transport of ions and metabolites [2,3]. Interestingly, the subunit structures of these enzymes are quite different. The subunit structure of vacuolar H^+ -translocating ATPase is generally more complicated but well characterized [1,2]. The vacuolar H^+ -ATPase consists of a peripheral protein complex and a transmembrane sector. The peripheral protein complex of vacuolar H^+ -ATPase contains three copies of both catalytic subunit A and regulatory subunit B as well as several other minor polypeptides. The transmembrane sector is composed of at least six copies of dicyclohexylcarbodiimide (DCCD)-binding proteolipid, which is believed to be the proton channel [1,2]. The molecular mass for vacuolar H^+ -ATPase was calculated in the range of 450–600 kDa. In contrast, vacuolar H^+ -PPase consists of only a single kind of polypeptide [3,4]. However, the molecular mass of substrate-binding vacuolar H^+ -PPase was determined as 73 or 66 kDa from mung bean [4,5], 67 kDa from red beet [6] and 37–45 kDa from maize [7] respectively. Furthermore, several workers deduced a molecular mass of approx. 81 kDa from the cDNA sequence for tonoplast pyrophosphatase of *Arabidopsis thaliana* [8], *Hordeum vulgare* [9] and *Beta vulgaris* [10]. Nevertheless, recent work resolved the controversy regarding the identity of the substrate-binding subunit and also determined the subunit as a polypeptide of approx. 66 kDa [4].

Despite all of the above-mentioned efforts, many investigators continue to dispute the nature of the subunit structure of tonoplast H^+ -PPase. On the one hand, previous works contended that tonoplast H^+ -PPase is a multimeric enzyme for PP_i hydrolysis

as determined by cross-linkage, gel filtration, and radiation inactivation analysis [7,11–14]. However, other laboratories [11,15] reported a monomeric functional mass of 88–91 kDa for substrate hydrolysis and proton translocation. The exact subunit structure and actual mechanism of vacuolar H^+ -PPase require further investigation.

This study determines the functional mass of vacuolar H^+ -PPase by radiation inactivation analysis. These results demonstrate that the vacuolar H^+ -PPase is *in situ* a dimer. Membrane solubilization and presence of ionophore, e.g. valinomycin + KCl, may alter its enzyme structure, thereby resulting in a different functional mass. A working model is also proposed to elucidate the subunit structure of vacuolar H^+ -PPase under various conditions.

MATERIALS AND METHODS

Membrane preparation

Seeds of *Vigna radiata* L. (mung bean), as obtained from a local market, were soaked overnight in tapwater and then germinated at room temperature in the dark. The hypocotyls of 4-day-old etiolated seedlings were excised, chilled on ice and then washed with distilled water. The sliced hypocotyls were ground with a Waring blender in a homogenization medium containing 0.25 M sorbitol, 5 mM EGTA, 1 mM PMSF, 1.5% (w/v) polyvinylpyrrolidone, 1% (w/v) ascorbic acid and 50 mM Mops/KOH (pH 7.6). The homogenate was filtered through a four-layer cheesecloth and then subjected to differential centrifugation at 3600 g for 10 min and at 120000 g for 20 min. The precipitate was resuspended in a solution (6 × 15 ml) containing 10 mM potassium phosphate (pH 7.8), 0.3 M sucrose, 1 mM EGTA and 2 mM dithiothreitol. The suspension was overlaid on a medium (6 × 15 ml) containing 5 mM Mops/KOH (pH 7.3), 0.25 M sorbitol, 1 mM EGTA and 2 mM dithiothreitol. After

Abbreviations used: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; H^+ -PPase, proton pyrophosphatase.

* To whom all correspondence should be addressed.

centrifugation at 120 000 *g* for 30 min, the interface portion (6 × 3 ml) was collected and diluted with a solution (6 × 30 ml) containing 5 mM Mops/KOH (pH 7.3), 0.25 M sorbitol, 1 mM EGTA and 2 mM dithiothreitol. The vacuolar-membrane vesicles were pelleted down at 130 000 *g* for 20 min; the resulting white precipitate was resuspended in a medium (6 × 3 ml) containing 20 mM Tris/Mes (pH 7.5), 20 % (v/v) glycerol, 1 mM dithiothreitol, 1 mM EGTA and 2 mM MgCl₂. The final protein concentration was approx. 1.5 mg/ml. The vesicle preparation was then stored at -70 °C until used.

Purification of H⁺-PPase

H⁺-PPase was purified from resealed tonoplast vesicles according to the method of Rea et al. with minor modifications [4]. The tonoplast vesicles were spun down at 120 000 *g* for 1 h and the pellet was redissolved in Buffer I (27 ml) containing 20 mM Tris/acetate (pH 7.5), 20 % (v/v) glycerol, 1 mM dithiothreitol, 1 mM Tris/EGTA, 2 mM MgCl₂ and a protein concentration of approx. 1 mg/ml. Sodium deoxycholate (2 mg/mg of protein) and KCl (50 mM) were added, respectively, and the vesicles incubated at 4 °C for 10 min. After centrifugation at 150 000 *g* for 30 min, the membrane pellet was resuspended in Buffer I (7 ml) and subjected to a second solubilization using 0.4 % (w/v) L- α -lysophosphatidylcholine for 30 min at room temperature. Following centrifugation at 150 000 *g* for 50 min, the supernatant (5–10 mg of protein) was concentrated to 0.2 ml and then applied to an FPLC column of Superose 12 HR 10/30 pre-equilibrated with a running buffer [20 mM Tris/HCl (pH 7.5), 20 % (v/v) glycerol, 0.1 % (w/v) Triton X-100, 1 mM Tris/EGTA, and 2 mM MgCl₂]. The flow rate was 0.4 ml/min at room temperature. Fractions (5 ml) with H⁺-PPase activity were collected and then applied on to a Mono Q HR 5/5 FPLC column pre-equilibrated with the same buffer as mentioned above. The H⁺-PPase was eluted at a flow rate of 0.5 ml/min by a salt gradient of 0–0.5 M NaCl. Fractions (1 ml) with the highest H⁺-PPase activity were collected for further studies.

Enzyme assay and protein determination

H⁺-PPase activities of membrane vesicles were determined by measuring the release of P_i from PP_i [16]. Since 2 mol of P_i are liberated from hydrolysis of 1 mol of PP_i, we have expressed the activity as mol of PP_i consumed. Aliquots (10 μ l) of resealed vesicles were assayed in a 1.0 ml reaction medium containing 25 mM Mops/KOH (pH 7.2), 3 mM MgSO₄, 50 mM KCl, 3 mM K₄PP₆, 16–20 μ g/ml membrane protein, 0.5 mM NaN₃, 0.1 mM sodium orthovanadate, 50 mM KNO₃ and 0.1 mM ammonium molybdate to inhibit possibly contaminated ATPases and alkaline phosphatases [2, 17]. When enzymic activity of the purified H⁺-PPase was measured, the reaction conditions were the same as above except that the inhibitors were excluded and the enzyme concentration was 2–5 μ g/ml. Assay was carried out at 33 °C for 15 min and terminated by adding a colour developing solution (2.0 ml) containing 1.7 % (w/v) ammonium molybdate, 2 % (w/v) SDS and 0.02 % (w/v) ANSA (1-amino-2-naphthol-4-sulphonic acid). The released P_i was determined spectrophotometrically as described elsewhere [17].

Protein concentration was determined according to a modified Lowry method [18] using BSA as a standard.

SDS/PAGE and gel-filtration chromatography

SDS/PAGE was performed as described by Laemmli [19]. The protein pattern on the gel was visualized by silver staining. Size-exclusion gel-filtration chromatography of vacuolar H⁺-PPase

was performed on a HPLC Shodex WS-804 column using a Shimadzu HPLC system. Purified enzyme (10–15 μ g) in 0.1 ml elution buffer was injected through the column and eluted by a buffer containing 50 mM Tris/HCl (pH 7.5), 100 mM K₂SO₄, 0.05 % (w/v) NaN₃ and 0.1 % (w/v) Triton X-100. The elution flow rate was 0.75 ml/min at room temperature. Molecular mass of native H⁺-PPase was determined using standard protein markers of thyroglobulin (669 kDa), apoferritin (443 kDa), α -amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa) and carbonic anhydrase (29 kDa).

Measurement of proton translocation

Proton translocation was measured as fluorescence quenching of Acridine Orange (excitation wavelength 495 nm, emission wavelength 530 nm) as described previously [16]. The reaction mixture (3 ml) contained 5 mM Mops/KOH (pH 7.9), 250 mM sorbitol, 3 mM MgSO₄, 50 mM KCl, 3 mM K₄PP₆, 0.1 mM sodium orthovanadate, 50 mM KNO₃, 0.1 mM ammonium molybdate, 0.5 mM NaN₃, 5 μ M Acridine Orange, and 16–20 μ g/ml membrane protein. The fluorescence quenching was initiated by adding 3 mM MgSO₄ (90 μ l). An aliquot (25 μ l) of the ionophore gramicidin (final concentration of 2 μ g/ml) was added at the end of each assay.

Irradiation and functional size determination

Samples were irradiated with ⁶⁰Co (~1000 Ci) as previously described [17, 20, 21]. The functional size of radiation inactivation was calculated according to the equation of Beauregard and Potier [22]:

$$\log m = 5.89 - \log D_{37,t} - 0.0028t$$

where *m* is the radiation inactivation size in Da and *D*_{37,*t*} is the dose in Mrad to bring the activity to 37 % of that of unexposed control at temperature *t* (°C) during irradiation. The functional size of the internal standard glucose-6-phosphate dehydrogenase (molecular mass = 104 kDa) was determined as previously described [23].

Chemicals

All chemicals were purchased from Sigma and Merck, and were used without further purification.

RESULTS AND DISCUSSION

First, we isolated the vacuolar H⁺-PPase from tonoplast of mung bean seedlings and then demonstrated that it contains only a single kind of polypeptide (Figure 1A). The molecular mass of the polypeptide is approx. 73 kDa as estimated by SDS/PAGE. The contamination is almost negligible in our preparation. The specific activity of purified vacuolar H⁺-PPase was approx. 150 μ mol of PP_i consumed/mg of protein per h, indicating at least a 15-fold purification from the membrane [4]. The purified vacuolar H⁺-PPase was then subjected to size-exclusion gel-filtration chromatography and a molecular mass of approx. 140 kDa was subsequently obtained (Figure 1B). The presence of Triton X-100 during enzyme preparation did not affect the determination of the molecular mass in our case (results not shown). Our result thus concurs with those of previous workers using chromatography [6] and disulphide cross-linking on polyacrylamide gel [13]. We therefore speculate that the purified vacuolar H⁺-PPase is probably a dimeric enzyme *in situ*.

An interesting question is raised as to whether native vacuolar H⁺-PPase is also dimeric on the vacuolar membrane. Radiation

Figure 1 SDS/PAGE pattern and size-exclusion gel filtration of the purified vacuolar H⁺-PPase

(A) SDS/PAGE pattern: lane 1, the standard markers with molecular masses indicated on the left; lane 2, purified vacuolar H⁺-PPase. (B) Size-exclusion gel-filtration chromatography of the vacuolar H⁺-PPase. H⁺-PPase (10 μ g) was injected through a Shodex protein WS-804F column. The column was equilibrated; and enzyme was eluted by a buffer containing 50 mM Tris/HCl (pH 7.5), 20 mM NaCl, 0.05% (w/v) NaN₃ and 0.08% (w/v) Triton X-100 with flow rate of 0.6 ml/min at room temperature. Carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), α -amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa) were used as molecular size standards. *, the peak maximum (140 kDa) of the H⁺-PPase.

inactivation is an effective technique for estimating the functional size of membrane-bound proteins and elucidating the structure and function of enzymes *in situ* [23–25]. Hence, this study performed radiation inactivation analysis to determine the molecular mass and to accurately predict the subunit structure of vacuolar H⁺-PPase both in membrane-bound and in the purified forms. To begin with, we verified whether this technique is feasible in our system by examining an internal standard, glucose-6-phosphate dehydrogenase [23,24]. Glucose-6-phosphate dehydrogenase (molecular mass, 104 kDa) was included in our sample solution and its radiation inactivation size measured accordingly. A D_{37} value of 7.26 ± 0.58 Mrad and calculated functional size of 109.2 ± 8.7 kDa were subsequently obtained (Figure 2, ○). This result clearly indicates that radiation inactivation analysis is effective under our conditions.

Upon exposure to high-energy irradiation, H⁺-PPase enzymic activity of vacuolar vesicles displayed a simple exponential decay with respect to the increase of dosage (Figure 2, ■). The

Figure 2 Radiation inactivation of membrane-bound and purified H⁺-PPases

The irradiation with ⁶⁰Co at -20 to -25 °C and the assay of PP_i hydrolysis were as described in the Materials and methods section. Each point was the mean from five independent experiments. From the linear regression, D_{37} values were 7.61 ± 0.59 Mrad for glucose-6-phosphate dehydrogenase (○), 5.62 ± 0.4 Mrad for tonoplast (■) and 10.9 ± 1.0 Mrad for purified H⁺-PPases (●) respectively.

dose–response relationship yielded a D_{37} value of 5.6 ± 0.4 Mrad. By target-size analysis, a functional size of 141.5 ± 10.8 kDa was calculated accordingly [22]. This value is similar to that obtained by the size-exclusion gel-filtration chromatography above (Figure 1B). The fact that vacuolar H⁺-PPase contains only one kind of polypeptide with a molecular mass of 73 kDa obviously revealed that tonoplast H⁺-PPase is a functional dimer *in situ*.

Furthermore, the functional size of purified vacuolar H⁺-PPase was also analysed here to observe whether they are the same in purified and membrane-bound states (Figure 2, ■). Upon exposure, purified vacuolar H⁺-PPase exhibited a similar response to gamma ray irradiation to that of vacuolar vesicles (Figure 2, ●). However, a larger D_{37} value of 10.9 ± 1.0 Mrad was unexpectedly obtained, thereby yielding a smaller functional size of 72.1 ± 11 kDa for the purified vacuolar H⁺-PPase. Molecular mass of the purified vacuolar H⁺-PPase is only one-half of that determined by size-exclusion gel-filtration chromatography and is in the dimension of its monomer (Figure 1B). From the above comparison, radiation inactivation analysis reveals different functional masses, thereby suggesting the possibility of distinct enzyme structures for purified and membrane-bound H⁺-PPases. This phenomenon is not unusual since many purified homo-multimeric enzymes have also displayed a monomeric functional size [24–26]. The functional size of these enzyme complexes indicates that monomeric subunit suffices to support their enzymic reactions even though they are structurally multimeric. We thus believe that solubilizing dimeric vacuolar H⁺-PPase from a membrane might induce a conformational change, subsequently allowing either only one of its subunits to express its enzymic activity or both subunits to function independently. The currently available technique could not distinguish between these two possibilities. Nevertheless, both cases yield an apparent monomeric functional size.

Figure 3 Radiation inactivation of PPase-mediated proton translocation

The irradiation with ^{60}Co at -20 to -25 °C and the assay of proton pumping were as described in the Materials and methods section. After the irradiation, proton transport was assayed as the fluorescence quenching in a medium containing 50 mM Tris/HCl (pH 7.0), 250 mM sorbitol, 50 mM KCl, 3 mM MgSO_4 , 5 μM Acridine Orange and 20 μg of tonoplast. Samples of 3 mM PP_i were added to initiate the proton translocation. The excitation wavelength was 495 nm, while the emission wavelength was 530 nm. Gramicidin (2.5 $\mu\text{g}/\text{ml}$) was added to terminate the reaction. (A) Reaction trace of fluorescence quenching by Acridine Orange. (B) Dose-response of H^+ -translocation rate. Initial rates of fluorescence quenching were determined from the change of the relative fluorescence during the first min. By linear regression, the D_{37} value was 4.96 ± 0.6 Mrad and molecular size was 158 ± 15 kDa. Each point was the mean from three independent experiments.

Pyrophosphate hydrolysis of tonoplast H^+ -PPase is coupled to proton pumping for secondary transport of metabolites into vacuoles [3]. Exactly how proton translocation is structurally associated with the enzymic reaction is of particular interest. We then determined the radiation inactivation size of H^+ -PPase-supported proton translocation of vacuolar vesicles using fluorescence quenching of Acridine Orange as the pH probe (Figure 3). The dose-dependent decay of fluorescence quenching upon irradiation was also in a simple exponential form, indicating a good association of proton translocation to the enzymic reaction (Figure 3B). The D_{37} value and functional size of H^+ -PPase-supported proton translocation were thus calculated as 4.9 ± 0.6 Mrad and 158.4 ± 19.5 kDa respectively. This functional size is quite similar to that of the enzymic activity of membrane-bound H^+ -PPase (141.5 ± 10.8 kDa). Consequently, we believe that two copies of substrate-binding polypeptide of vacuolar H^+ -

Figure 4 Effects of uncouplers and ionophore on functional size of H^+ -PPase

After irradiation, 50 μM uncouplers (gramicidin and FCCP) or 2 μM valinomycin (+50 mM KCl) were added in a reaction medium as described in the Materials and methods section. D_{37} values of 4.54 ± 0.7 , 4.70 ± 0.3 , and 12.2 ± 1.2 Mrad were obtained in the presence of gramicidin (●), FCCP (△), and valinomycin + KCl (■) respectively. Each value was the mean of six independent assays.

Table 1 Functional size of H^+ -PPase as determined in the presence of uncouplers and ionophores

	D_{37} (Mrad)	Functional size (kDa)
Proton translocation	4.96 ± 0.61	158.4 ± 19.5
Enzymic activity		
(A) Membrane-bound H^+ -PPase		
Control	5.62 ± 0.43	141.5 ± 10.8
+ Gramicidin	4.54 ± 0.74	175.0 ± 28.5
+ FCCP	4.70 ± 0.32	162.9 ± 11.1
+ Valinomycin	12.20 ± 1.21	63.4 ± 6.3
(B) Purified H^+ -PPase	10.90 ± 1.02	71.1 ± 6.7

PPase are associated in a dimeric form to exert the enzymic and proton-pumping reactions of the vesicles.

Uncouplers and ionophores are the membrane perturbation factors which disrupt the pH gradient and the electrochemical potential across the membrane [27]. We therefore examined the possible effects of uncouplers and ionophores so as to determine the functional size of H^+ -PPase on the vacuolar membrane. Vacuolar vesicles were subjected to irradiation and then enzymic activities were measured in the presence of the uncouplers and ionophores. Figure 4 shows that the enzymic activity of vacuolar H^+ -PPase also decayed as a simple exponential function of dose in the presence of ionophores and uncouplers. D_{37} values of 4.54 ± 0.7 , 4.7 ± 0.3 and 12.2 ± 1.2 Mrad were obtained for the vacuolar H^+ -PPase in the presence of gramicidin, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) and valinomycin + KCl, respectively. Table 1 summarizes the calculated functional masses of the vacuolar H^+ -PPase in the presence

Figure 5 A working model for the subunit structure of vacuolar H⁺-PPase

(A) Solubilization of vacuolar H⁺-PPase from vesicle membrane; (B) depletion of membrane potential by valinomycin (val.) + KCl.

of various uncouplers and ionophores. No significant difference in the functional size of PP_i hydrolysis was found when uncouplers such as gramicidin and FCCP were present while measuring the enzymic activity. However, as ionophore (valinomycin and KCl) was added in the reaction medium, the functional size of membrane-bound H⁺-PPase was decreased to one-half of that in its absence. A similar perturbation by ionophores was also found when determining the functional size of other proton-pumping enzymes, e.g. vacuolar, thylakoid and mitochondrial H⁺-ATPases (results not shown). We thus believe that the structure and function of H⁺-PPase are sensitive to its membrane environment, thereby resulting in the different functional sizes, as in this case when ionophore (valinomycin and KCl) is present.

Based on these considerations, a working model is proposed for the phenomenon observed above (Figure 5). Vacuolar H⁺-PPase on the membrane is a homodimer (I) with a molecular mass of 73 kDa for its single subunit. The enzymic activity is well associated with proton pumping, thereby resulting in a functional size of 140 kDa for both reactions. Vesicle membrane provides an appropriate environment for vacuolar H⁺-PPase to maintain its native structure and, consequently, to exert the enzymic activity and proton translocation. Upon depletion of the membrane (Pathway A), the subunit structure of the vacuolar H⁺-

PPase remains as a dimer, but is concomitant with a conformational change of the individual subunit (II). The functional size of the vacuolar H⁺-PPase in a purified form (71.1 ± 6.7 kDa) is thus one-half of that on the membrane; it is also similar to the molecular mass of the individual polypeptide. Furthermore, the native structure of vacuolar H⁺-PPase is extremely sensitive to the electrochemical potential but not to the pH gradient across the membrane. As the membrane potential is dissipated by the ionophore, e.g. valinomycin and KCl (Pathway B), the architecture of vacuolar H⁺-PPase complex is changed. Consequently, each subunit independently possesses the hydrolysis activity, thereby resulting in a monomeric functional size (III).

This work was supported by a grant from the National Science Council, Republic of China (NSC84-2311-B007-008) to R. L. P.

REFERENCES

- Nelson, N. and Taiz, L. (1989) *Trends Biochem. Sci.* **14**, 113–116
- Sze, H. (1985) *Annu. Rev. Plant Physiol.* **36**, 175–208
- Rea, P. A. and Poole, R. J. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 157–180
- Rea, P. A., Britten, C. J. and Sarafian, V. (1992) *Plant Physiol.* **100**, 723–732
- Maeshima, M. and Yoshida, S. (1989) *J. Biol. Chem.* **264**, 20068–20073
- Sarafian, V. and Poole, R. J. (1989) *Plant Physiol.* **91**, 34–38
- Chanson, A. and Pilet, P. (1989) *Plant Physiol.* **90**, 934–938
- Sarafian, V., Kim, Y., Poole, R. J. and Rea, P. A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1775–1779
- Tanaka, Y., Chiba, K., Maeda, M. and Maeshima, M. (1993) *Biochem. Biophys. Res. Commun.* **181**, 962–967
- Kim, Y., Kim, E. J. and Rea, P. A. (1994) *Plant Physiol.* **106**, 375–382
- Sarafian, V., Potier, M. and Poole, R. J. (1992) *Biochem. J.* **283**, 493–497
- Britten, C. J., Turner, J. C. and Rea, P. A. (1989) *FEBS Lett.* **256**, 200–206
- Maeshima, M. (1990) *Biochem. Biophys. Res. Commun.* **168**, 1157–1162
- Sato, M. H., Maeshima, M., Ohsumi, Y. and Yoshida, M. (1991) *FEBS Lett.* **290**, 177–180
- Fraichard, A., Magnin, T. and Rugin, A. (1991) *Plant Physiol.* **96**, S-159
- Kuo, S. Y. and Pan, R. L. (1990) *Plant Physiol.* **93**, 1128–1133
- Wang, M. Y., Lin, Y. H., Chow, W. M., Chung, T. P. and Pan, R. L. (1989) *Plant Physiol.* **90**, 475–481
- Larson, E., Howlett, B. and Jagendorf, A. T. (1986) *Anal. Biochem.* **155**, 243–248
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Wu, J. J., Ma, J. T. and Pan, R. L. (1991) *FEBS Lett.* **283**, 57–60
- Ma, J. T., Wu, J. J., Tzeng, C. M. and Pan, R. L. (1993) *J. Biol. Chem.* **268**, 10802–10807
- Beauregard, G. and Potier, M. (1985) *Anal. Biochem.* **150**, 117–120
- Pan, R. S., Chien, L. F., Wang, M. Y., Tsai, M. Y., Pan, R. L. and Hsu, B. D. (1987) *Plant Physiol.* **85**, 158–163
- Kempner, E. S. and Schlegel, W. (1979) *Anal. Biochem.* **92**, 2–10
- Kempner, G. R. and Macey, R. I. (1968) *Biochim. Biophys. Acta* **163**, 188–203
- Kempner, E. S. (1988) *Adv. Enzymol.* **66**, 107–147
- Pressman, B. C. (1976) *Annu. Rev. Biochem.* **45**, 501–529