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Effects of high night temperature on lipid and protein compositions in tonoplasts isolated from *Ananas comosus* and *Kalanchoë pinnata* leaves

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Abstract

Effects of high night temperature on the lipid and protein compositions in the tonoplasts isolated from the leaves of two Crassulacean acid metabolism (CAM) plants, *Ananas comosus* (pineapple) and *Kalanchoë pinnata* were studied. The results showed that the phospholipids/protein ratios in the tonoplasts isolated from pineapple and *K. pinnata* leaves decreased from 1.82 to 1.21 and 2.63 to 1.50, respectively, as the night temperature increased from 20 to 37 ºC. Under high night temperature, relative amount of total unsaturated fatty acids in *K. pinnata* was increased by 6 %, which was mainly caused by increased C18:2 and C18:3, whereas unsaturated fatty acids, C18:2 and C18:3 in pineapple did not show significant change. The distribution patterns of tonoplast proteins in the two CAM species were different between normal and high night temperature and in *K. pinnata*, especially those with molecular mass ranging from 66.2 to 97.4 KDa. Compared with normal night temperature, more proteins were found in pineapple, but no difference was found in *K. pinnata*. Thus, above result indicated that the pineapple tonoplasts could keep higher rigidity under high night temperatures compared to the *K. pinnata*.

*Additional key word*s: CAM, fatty acids, malate, pineapple, tonoplast fluidity.

Introduction

In Crassulacean acid metabolism (CAM) plants, CO_2 is fixed *via* phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) in dark. The malate formed is then accumulated to high concentration in the vacuole. Recently, we investigated the effects of high night temperature on CO_2 exchange and organic acid accumulation in two CAM species, *Kalanchoë pinnata* and *Ananas comosus* (pineapple) with the day temperature maintained at 30 ºC. The results showed that *K. pinnata* lost nocturnal CO_2 uptake completely at night temperature of 37 ºC, whereas pineapple kept significant nocturnal malate accumulation in the vacuole (Lin *et al*. 2006). It has been assumed that the ability to accumulate malate into the vacuole in the dark depends on the actual fluidity of the tonoplast and that effective nocturnal malate storage in CAM plants is facilitated by a relatively rigid tonoplast (Kluge *et al*. 1991, Kliemchen *et al*. 1993). Behzadipour *et al*. (1998) found that exposure of *Kalanchoë daigremontiana* plants to higher growth temperature compared with the control decreased the fluidity of tonoplast. However, Wilkins (1983) reported that high temperatures enhance the release of malate from the vacuole by opening the "gate" in the tonoplast. Therefore, it is still equivocal about the response of tonoplast fluidity of CAM plants to high night temperature. The tonoplast mainly consists of a lipid-protein bilayer. The change in the tonoplast lipid and protein compositions would inevitably alter tonoplast fluidity and affect nocturnal malate accumulation in the vacuole. To our best knowledge, such data are very limited for CAM plants (Behzadipour *et al.* 1998).

The objective of this study is to understand the possible influence of tonoplast compositions on nocturnal malate accumulation in the vacuole of CAM plants. For this purpose, we investigated lipid and protein compositions and contents in the tonoplasts isolated from the leaves of two CAM plants, pineapple and *K. pinnata* under normal and high night temperatures.

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*Abbreviation*s: ATPase - adenosinetriphosphatase; CAM - Crassulacean acid metabolism; 2-DE - two-dimensional gel electrophoresis; PPase - inorganic pyrophosphatase; PEPC - phospho*enol*pyruvate carboxylase; PAR - photosynthetically active radiation.
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Materials and methods

Plants: *Ananas comosus* (L.) Merr. cv. Smooth-cayenne N67-10 (pineapple) and *Kalanchoë pinnata* (Lam.) Pers. were vegetatively propagated and grown in pots in a greenhouse under natural photoperiod. Plants were transferred to a growth chamber (*KG-50 HLA*, *Koito Industrial Co.*, Yokohama, Japan) with day/night temperature of 30/20 ºC for normal and 30/37 ºC for high night temperature. The relative air humidity (RH) was 70 % and PAR 420 to 450 μmol m^{-2} s^{-1} at the mid-plant height during photoperiod. After one or two weeks, fourth to eighth leaf pairs, counting from the apex of *K. pinnata* and fully expanded mature leaves of pineapple were used in experiments.

Tonoplast isolation: Tonoplasts were isolated according to Chen and Nose (2000). The leaf midrib and margins of *K. pinnata*, and the base and top of pineapple leaves were removed. About 100 g tissue was homogenized with a blender (*MX-X1*, *National Elect. Co.*, New Hartford, USA) in 200 cm^3 buffer containing 450 mM mannitol, 3 mM $MgSO_4$, 10 mM ethylene glycol-bis(aminoethyl-ether)-*N*,*N*,*N*,*N*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 % (m/v) bovine serum albumin (fraction V), 100 mM *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl] glycine (Tricine), adjusted to pH 8.0 with 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris). The homogenate was filtered though two layers of cheesecloth. The filtrates were centrifuged at 8 095 *g* (*P28S* rotor, *CP75* ultracentrifuge, *Hitachi Koki Co*., Takeda Katsuta, Japan) for 15 min. The supernatant was layered over a 25 % (m/v) sucrose cushion containing 1 mM DTT and 5.0 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (Hepes), adjusted to pH 8.0 with Tris. The gradients were centrifuged at 103 872 *g* for 1 h. The membrane vesicles were removed from the interface using a Pasteur pipette and diluted 1:1 (v/v) with a medium containing 150 mM mannitol, 1 mM DTT and 25 mM 1,3-bis[tris (hydroxy-methyl) methylamiol] propane (BTP), adjusted to pH 8.0 with 2-(N-morpholino) ethanesulfonic acid (Mes). The vesicles were then pelleted by centrifugation at 103 872 *g* for 30 min. Finally, the pellets were resuspended in the 4 cm^3 medium containing 150 mM mannitol, 1mM DTT and 25 mM BTP-Mes. All fraction steps were performed at 4 ºC.

Analysis of tonoplast ATPase: ATPase activity was assayed according to the method of Chen and Nose (2000) with some modifications. Most of ATPase in the membrane were sensitive to nitrate and bafilomycin A_1, inhibitors of vacuolar ATPase, but insensitive to azide and vanadate, inhibitors of mitochondrial and plasmalemma. The enzyme activity was assayed for 30 min in a 0.5 cm^3 reaction mixture containing 50 mM BTP-Mes, pH 8.0, 3 mM Na_2-ATP, 3 mM $MgSO_4$, 0.02 % (m/v) Triton X-100, 1 mM sodium molybdate and 50 mM KCl. The reaction started with the addition of 0.05 cm^3 of sample, and stopped with the addition of 0.25 cm^3 of 6 % (m/v) sodium dodecyl sulfate (SDS) (assay temperature). The released Pi from the substrate was determined according to the method of Chen and Nose (2000). ΔNO_3^--ATPase and ΔVO_3^--ATPase were determined as the activity inhibited by 100 mM KNO_3 and 0.5 mM NH_4VO_3, respectively (Δ means the remainder between the actual activities and inhibited activities).

Lipid analysis: The total lipid was extracted and purified according to the method of Bligh and Dyer (1959). 4.5 cm^3 2:1 (v/v) of chloroform and methanol was added to a test tube containing 1.5 cm^3 tonoplast extraction. After vortexing, 1.5 cm^3 chloroform was added to the mixture. The tube was then kept in 37 ºC bath for 1 h. Afterwards, some water was added to the mixture and finally its volume ratio of the chloroform:methanol:water was maintained at 1:1:0.8. After centrifugation at 3 000 *g* for 15 min, the lower chloroform layer was collected, and then concentrated by nitrogen gas. The remained solid matter was dissolved by petroleum ether and was used for lipid analysis.

The tonoplast fatty acid compositions were determined using a gas-liquid chomatography (*GC14A* and *Omegawax*-320 column; 30 m length, 0.25 mm diameter, *Shimazu*, Tokyo, Japan). Both the injector and flame ionization detector (FID) temperature was 250 ºC, and column temperature was 200 ºC. Flow rate of carry gas (He) was 2.4 cm^3 min^{-1} and the split ratio was set as 1/50.

Protein assay: The tonoplast protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

For two-dimensional (2-DE) assay, proteins were extracted from purified tonoplast of pineapple and *K. pinnata*. In order to prevent tonoplast protein from decomposition, tonoplast was isolated in a container filled with nitrogen gas prior to the test. After the tonoplast was isolated, in order to prevent the resolution of tonoplast protein from the influence of lipid (manufacturer's instruction of *Amersham Biosciences*, Geneva, Switzerland), the prepared tonoplast was firstly added to 90 % (v/v) ice-cold acetone, mixed by vortexing and stored for 5 min at 4 ºC. Pellets were collected by centrifuging at 18 000 *g* for 5 min. The supernatant was discarded. The pellets were washed 3 times with 90 % acetone. The final pellets were dissolved in 2-DE sample buffer containing 9.8 M urea, 2 M thiourea, 40 mM trizma-base, 2 % (m/v) 3-[3-(cholamidopropyl) dimethyl-ammonio]-1-propane-sulfonate (CHAPS), 2 M trisbutyl-phosphine (TBP) and 0.5 % (m/v) IPG buffer and four types of protease inhibitor, 0.2 mM monoiodoacetate (MIA), 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM *p*-chloromercuribenzoate (PCMB) and 0.1 mM leupeptin.

2-DE was performed according to the recommendation of 2-DE manual (*Amersham Biosciences*). All experiments were performed with nonlinear immobilized

pH gradient strips (*NL-IPGs*) with a pH range of 3 - 10. For preparation of 2-DE, the swell solution as an aliquots of 0.34 cm^3 containing 8 M urea, 0.5 % (m/v) CHAPS, 2 % (m/v) IPG buffer (3 - 10), 0.2 % (m/v) DTT and 0.002 % (m/v) bromophenol blue without sample was added. The swelling was continued for 10 - 15 h by using a dried 180-mm, pH range of 3 to 10 nonlinear immobilized pH gradient strips according to the manufacturer instruction (*Immobiline DryStrips*, *Amersham Pharmacia Biotech.*, Uppsala, Sweden). Isoelectric focusing (IEF) was performed for 1 min at 300 V, 1.5 h at 300 - 3500 V gradient, and then held at 3500 V for 4.5 h at 20 ºC on a flat-bed eletrophoresis unit (*Multiphor II* system, *Amersham Pharmacia Biotech*.) with 30 μg tonoplast protein that was resuspended in a 2-DE sample buffer (as described in the part of protein extraction). Immobilized pH gradient strips were then transferred to 10 cm^3 of an equilibration buffer consisting of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30 % (m/v) glycerol, 2 % (m/v) SDS, 0.002 % (m/v) bromophenol blue, and 100 mg DTT for the first equilibration step and 250 mg iodoacetamide for the second equilibration step. The strips were incubated for 15 min with vibration. The equilibrated strips were slotted at 15 ºC into an *ExcelGel* SDS Gradient 12 - 14 which performed for 35 min at 600 V, 20 mA of first step and 1.5 h at 600 V, 50 mA of second step.

The separated proteins on the 2D-eletrophosis gels were stained with *Sypro Ruby* (*Bio-Rad*, Hercules, CA, USA). The protein spots were detected using a *Typhoon 9000E* (*Amersham Bioscience*). The results were processed with the *imageMaster 2D* platinum software (*Amersham Bioscience*).

Results

The activities of extracted membrane ATPase were largely inhibited by the nitrate, whereas it was not inhibited by vanadate either under the normal or high night temperature condition (Table 1). These results indicated that the membrane fraction used in the analysis of phospholipids and proteins was only slightly contaminated by plasmalemma membranes. This finding is consistent with Kliemchen *et al*. (1993). In addition, tonoplast ATPase activity at both 20 and 37 ºC night temperatures was higher in the pineapple than in the *K. pinnata*. Similar result has been obtained by Chen and Nose (2000).

Table 1. ATPase activity [μmol(Pi) mg^{-1}(protein) h^{-1}] as affected by 100 mM nitrate (inhibitor for tonoplast ATPase) and 0.5 mM vanadate (inhibitor for plasmalemma ATPase). NT, day/night temperature 30/20 ºC; HT, day/night temperature was 30/37 ºC) for one week. Means ± SE, $n = 6$. In parentheses % of control.

Species	Control		ΔNO_3-ATPase		ΔVO_3-ATPase	
	NT	HT	NT	HT	NT	HT
Pineapple	25.3 ± 1.3	57.1 ± 1.6	20.9 ± 1.3 (82.8)	45.1 ± 2.2 (78.9)	3.0 ± 0.4 (12.0)	6.8 ± 0.6 (11.9)
K. pinanta	19.7 ± 0.5	38.5 ± 0.5	15.5 ± 0.4 (78.5)	31.0 ± 0.4 (80.4)	2.5 ± 01 (13.3)	5.0 ± 0.6 (12.9)

Table 2. The contents of phospholipids and proteins [g dm^{-3}] and their ratio in the isolated tonoplast of pineapple and *K. pinnata* at the normal and high night temperature condition. *, ** - significant differences between NT and HT in same plant at $P < 0.01$ and 0.001, respectively; #, ## - significant differences between pineapple and *K. pinnata* in same temperature at $P < 0.05$ and 0.01, respectively.

Species		Phospholipids	Proteins	Ratio
Pineapple	NT	0.40 ± 0.01	0.22 ± 0.01	1.82 ± 0.05
	HT	0.26 ± 0.02**	0.21 ± 0.03	1.21 ± 0.08**
K. pinnata	NT	0.51 ± 0.07#	0.19 ± 0.02#	2.63 ± 0.13##
	HT	0.26 ± 0.03*	0.17 ± 0.02	1.50 ± 0.12**#

When night temperature increased from 20 to 37 ºC, phospholipid content decreased in the tonoplast of pineapple and *K. pinnata* (Table 2). Phospholipid content in pineapple was 22 % lower than in *K. pinnata* under the normal night temperature. However, phospholipid contents in two plants were almost the same under the high night temperature. In contrast, protein content in the tonoplast was less affected by high nigh temperature. Protein content was slightly higher in pineapple than in *K. pinnata* under both the normal and high night temperatures. Under high night temperature, phospholipid/protein ratio in the tonoplasts of pineapple and *K. pinnata* was decreased by 33.5 and 42.9 %, respectively. The decrease was mainly caused by the reduction of phospholipid fraction.

As night temperature increased from 20 to 37 ºC, percentage of total unsaturated fatty acids in *K. pinnata* increased from 47.8 to 53.2 %, which was mainly caused by the increased C18:2 and C18:3, whereas total unsaturated fatty acids, C18:2 and C18:3 in pineapple did not show significant change (Table 3). High night temperature resulted in a decrease in C14:0 and C16:0 in the two CAM species, C18:0 and C18:1 in pineapple, but had no obvious influence on other fatty acids in the two

CAM species.

About 337 proteins were found under the normal night temperature in pineapple. Most of the tonoplast proteins were located with a pH range of 5 to 9. The molecular mass of about 66 % proteins was between 21.5 to 66.2 kDa and only 5 % proteins were detected between 66.2 to 97.4 kDa. About 380 proteins were detected under the high night temperature. Compared with the normal night temperature, some spots of proteins disappeared and some became bigger and clearer compared with the normal night temperature (Fig. 1). About 278 proteins were found at the normal night temperature in *K. pinnata*. Most of them were between 31.0 to 97.4 kDa. Compared to pineapple, the amounts of proteins were less, but some higher molecular mass (66.2 to 97.4 kDa) proteins were found. About 81 % proteins were distributed between 21.5 to 66.2 kDa. and 11 % between 66.3 to 97.4 kDa. Under the high night temperature, about 279 proteins were obtained from the 2-DE PAGE protein analysis. Compared to the normal night temperature, many spots of high molecular mass proteins became smaller or disappeared (Fig. 2).

The isoelectric points and molecular masses of pineapple and *K. pinnata* proteins were compared with the known data of isoelectric points and molecular mass in the tonoplast proteins of *Arabidopsis thaliana* (Carter *et al*. 2004) (Table 4). PPase was not found in the two CAM species in our results. Nine and eight types of transporters related to ATPase were found in the tonoplast of pineapple and *K. pinnata*, respectively. Some transporter proteins related to ATPase were found in the two CAM species but others were only found in one.

Table 3. Fatty acid composition of tonoplast isolated from pineapple and *K. pinnata*: C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), C16:1 (palmitoleic acid), C18:1 (oletic acid), C18:2 (linoleic acid) and C18:3 (linolenic acid).

Species		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Pineapple	NT	3.3 ± 0.2	31.5 ± 0.4	3.6 ± 0.3	6.7 ± 0.3	23.6 ± 0.2	23.3 ± 0.6	8.1 ± 0.2
	HT	1.3 ± 0.4	35.1 ± 1.3	0.5 ± 0.0	6.3 ± 1.0	25.2 ± 3.6	22.2 ± 1.2	10.2 ± 1.4
K. pinnata	NT	5.5 ± 1.7	34.5 ± 0.5	4.4 ± 1.4	12.2 ± 2.9	14.4 ± 1.8	24.2 ± 1.2	4.8 ± 1.2
	HT	1.8 ± 0.1	37.4 ± 0.9	0.4 ± 0.4	7.3 ± 0.7	2.8 ± 0.5	40.1 ± 0.7	10.2 ± 0.3

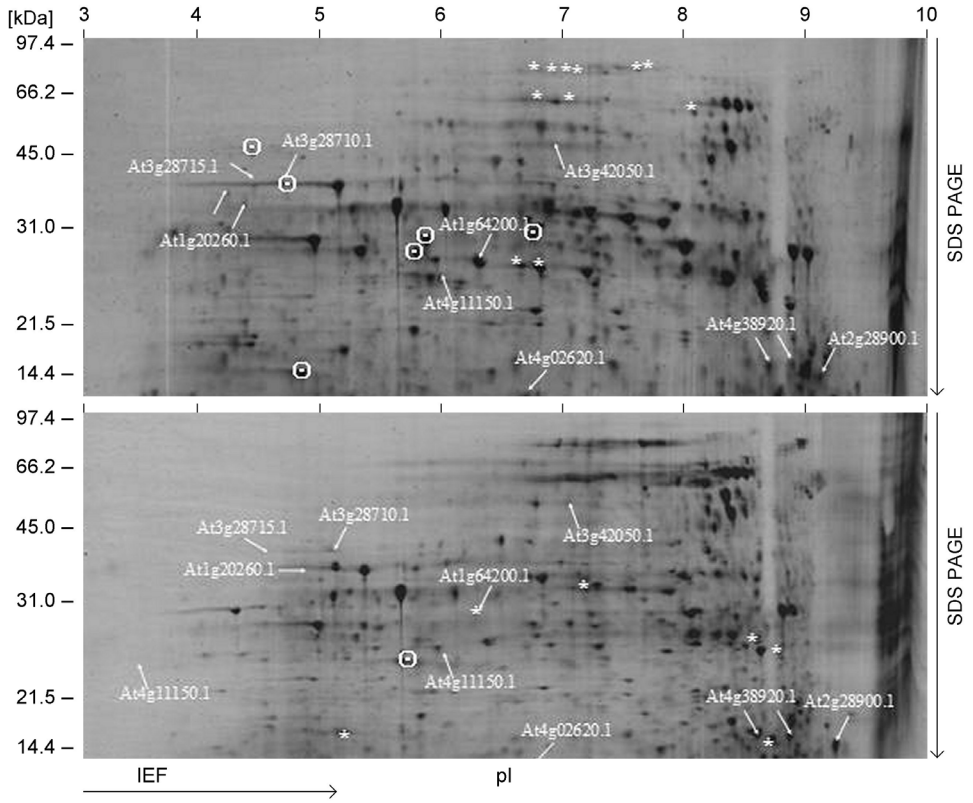

Fig. 1 The Sypro Ruby stained 2-DE maps of tonoplast proteins in the leaves of pineapple at the normal (*upper*) and high (*lower*) night temperature. The protein solution includes four kinds of protease inhibitors, 0.2 mM MIA, 0.5 mM PMSF, 1 mM PCMB and 0.1 mM leupeptin. *Circle with dot* represents the disappeared protein spots and *asterisk* represents the reduced protein spots which distinguished by eyes compared to another 2-DE PAGE. The name of every spot which is marked in the figure was cited from Carter *et al*. (2004).

Table 4. The putative functional categorization of tonoplast proteins in the two kind of CAM plants, pineapple and *K. pinnata*. The PI and Mr of proteins in our results were conjectured with the known data of the tonoplast of *Arabidopsis thaliana* (Carter *et al*. 2004), which was calculated with 5 % of error (- the protein not found).

Locus	Description	Pineapple				*K. pinnata*			
		NT		HT		NT		HT	
		PI	Mr	PI	Mr	PI	Mr	PI	Mr
At1g12840.1	vacuolar ATP synthase subunit C-related	-	-	-	-	5.44	44691	5.31	41704
At1g15690.1	inorganic pyrophosphatase-related	-	-	-	-	-	-	-	-
At1g20260.1	vacuolar H^+-ATPase subunit B-related	4.24	36511	4.83	36617	4.75	33190	4.68	34746
		4.39	34260						
At1g64200.1	H^+-transport ATPase protein related	6.36	26810	6.25	28290	6.08	28032	6.23	25817
						5.89	27904	6.24	28216
At1g76030.1	vacuolar ATP synthase subunit B	-	-	-	-	-	-	-	-
At1g78900.1	ATPase 70-kD subunit-related	-	-	-	-	-	-	-	-
At2g21410.1	vacuolar proton-ATPase subunit-related	-	-	-	-	-	-	-	-
At2g28900.1	membrane channel protein-related	9.22	15888	9.24	16063	9.62	15434	9.94	14751
At3g01390.2	vacuolar membrane ATPase subunit G (AVMA10)	-	-	-	-	-	-	-	-
At3g28710.1	adenosine triphosphatase-related, similar to vacuolar adenosine triphosphatase subunit D	4.71	39264	5.06	40916	-	-	-	-
At3g28715.1	expressed protein, similar to vacuolar adenosine triphosphatase subunit D	4.47	38909	4.51	40272	-	-	-	-
At3g42050.1	vacuolar ATP synthase subunit H-related	7.00	47683	7.03	52679	7.19	52852	7.20	51179
At4g02620.1	V-ATPase-related	6.76	14.509	6.71	14400	6.10	15316	6.74	14869
At4g11150.1	H^+-transporting ATPase chain E, vacuolar	6.03	25005	6.36	26213	6.20	25817	6.35	25415
						6.37	25233	6.04	24630
At4g23710.1	V-ATPase subunit G (vag2 gene)	-	-	-	-	-	-	-	-
At4g38510.1	Protable H^+-transporting ATPase	-	-	-	-	-	-	-	-
At4g38920.1	H^+-pumping ATPase 16-kD proteolipid, vacuole (ava-p1)	8.98	17007	8.58	17282	8.96	17460	8.93	16904
		8.80	16879	8.86	16904	9.06	17061	8.79	16502
At4g39080.1	proton pump-related vacuole proton ATPase 100-kD subunit	-	-	-	-	-	-	-	-

As shown above, large amounts of protein spots of *K. pinnata* became disappeared and reduced as night temperature increased from 20 to 37 °C. In contrast, in pineapple, the protein spots became bigger or clearer when the night temperature rose to 37 °C. Also, some protein spots of pineapple disappeared. Therefore, the changes in night temperature could have influence on the distribution patterns and types of tonoplast proteins.

Discussion

Our results showed that high night temperature resulted in a decrease in phospholipids/proteins ratio in the two CAM species. Similar result has been obtained by Kliemchen *et al*. (1993). Behzadipour *et al*. (1998) found that exposure of *K. daigremontiana* plants to higher growth temperature compared with the control decreased the fluidity and lipid/protein ratio of tonoplast. It could be suggested that pineapple and *K. pinnata* tonoplasts became more rigid with increasing night temperature. Phospholipids/proteins ratio in the tonoplasts of pineapple was always lower than in *K. pinnata* under both the normal and high night temperatures, suggesting that pineapple tonoplasts were kept in a more rigid state compared with *K. pinnata* either under the normal or high night temperatures. It has been suggested that malate influx into vacuoles of CAM plants is active, and malate efflux is passive, and effective malate accumulation in the vacuoles can occur only if the rate of passive malate efflux out of the vacuoles remains low with respect to rate of active influx (Lüttge and Smith 1984, Smith *et al*. 1996). Obviously, nocturnal malate storage in CAM plants is facilitated by a relatively rigid tonoplast (Kluge *et al*. 1991, Kliemchen *et al*. 1993). Thus, higher tonoplast rigidity in pineapple compared with *K. pinnata* may have contributed to the difference of nocturnal malate accumulation between the two CAM species.

Membrane fluidity is highly influenced by fatty acid compositions, especially the relative amounts of unsaturated fatty acid (Steponkus 1981). It has been known that the phospholipids containing longer-chained unsaturated fatty acid could improve the tonoplast fluidity. Our results showed that the relative amount of total unsaturated fatty acids in pineapple tonoplasts was kept stable, whereas about 6 % increase was found in the

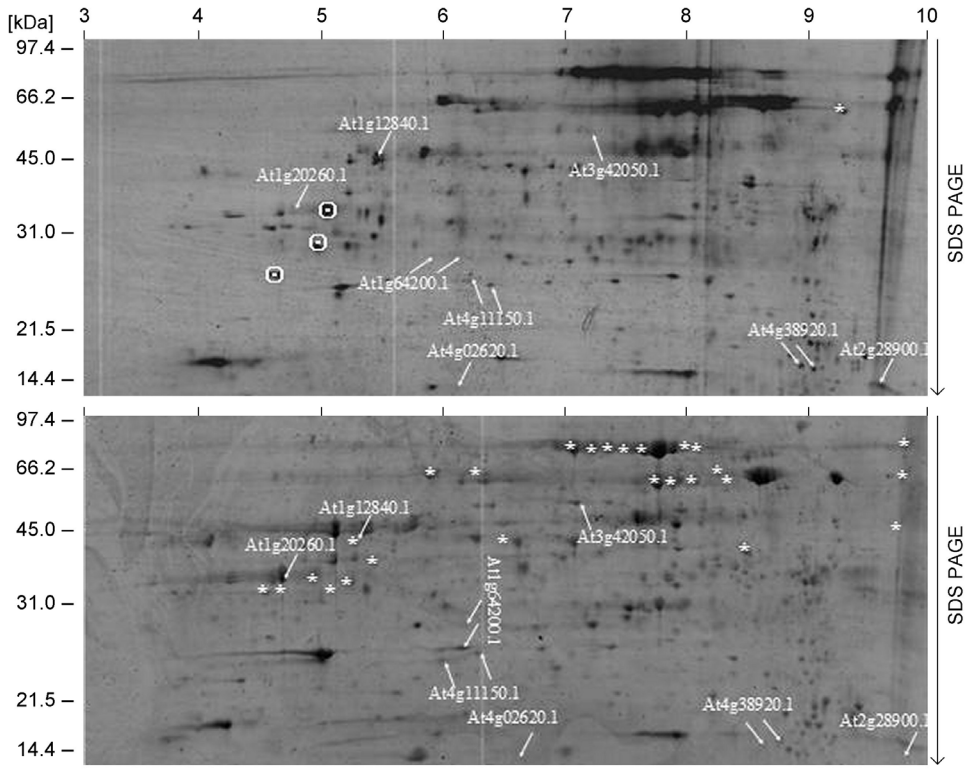

Fig. 2. The Sypro Ruby stained 2-DE maps of tonoplast proteins in the leaves of *K. pinnata* at the normal (*upper*) and high (*lower*) night temperature. The protein solution includes four kinds of protease inhibitors, 0.2 mM MIA, 0.5 mM PMSF, 1 mM PCMB and 0.1 mM leupeptin. *Circle with dot* represents the disappeared protein spots and *asterisk* represents the reduced protein spots which distinguished by eyes compared to another 2-DE PAGE. The name of every spots which marked in the figure was cited from Carter *et al*. (2004).

K. pinnata when the night temperature increased from 20 to 37 °C. It has been known that unsaturated fatty acids increase membrane fluidity. Thus, the increased unsaturated fatty acids in *K. pinnata* could enhance tonoplast fluidity and suppressed its rigidity or promoted malate efflux from vacuole to a certain extent compared with pineapple. As shown in the results, ATPase activity in pineapple tonoplasts was higher than that in *K. pinnata* one under both normal and high night temperatures, suggesting that the tonoplast ATPase of pineapple might provide more driving force for malate accumulation inthe photosynthetic cells than that of *K. pinnata*. Based on the results and analysis mentioned above, it could be suggested that under high night temperature pineapple is more able to store malate through enhancing malate

Table 5. The list of some proteins which could be distinguished by eyes become disappeared and reduced compared between normal and high night temperature condition in the same plant of pineapple and *K. pinnata.* - represents the spots shown in the 2-DE PAGE became disappeared compared with different temperature condition in the same plant. * represents the spots shown in the 2-DE PAGE became reduced compared with different temperature condition in the same plant. The locations of these spots also were marked in the Figs. 1 and 2.

Pineapple				*K. pinnata*			
NT		HT		NT		HT	
PI	Mr	PI	Mr	PI	Mr	PI	Mr
*		7.08	77399	7.40	78114	*	
*		7.33	77399	7.09	77399	*	
*		7.50	77399	7.60	75989	*	
*		7.64	77399	7.25	75989	*	
*		7.79	77399	7.85	77399	*	
*		6.94	77399	8.09	76691	*	
*		7.20	76691	8.20	76691	*	
*		8.15	59756	8.73	76691	*	
*		5.35	35756	9.93	76691	*	
*		5.65	24166	8.72	63082	*	
*		6.82	31000	8.09	61578	*	
-		8.72	16539	8.24	63082	*	
6.87	26194	*		8.38	64622	*	
6.82	30113	-		9.73	62576	*	
4.45	46322	-		6.40	62576	*	
5.92	29421	-		7.63	61578	*	
4.87	16252	-		5.98	64104	*	
4.71	39264	-		7.78	61085	*	
5.83	27761	-		5.02	33910	-	
				4.94	28944	-	
				4.60	26173	-	

influx from cytosol and decreasing its leakage from vacuole compared with *K. pinnata*.

Our results showed that exposure of pineapple to 37 °C night temperature compared with the normal night temperature increased 43 protein contents, but the amounts of proteins in *K. pinnata* were kept unchanged with increasing night temperature. Molecular masses of most proteins were less in pineapple tonoplast than in *K. pinnata* tonoplast. However, the amounts of proteins in pineapple tonoplasts were higher than that in *K. pinnata* ones. In *K. pinnata*, many spots of high molecular mass proteins became lower or disappeared under high night temperature (Table 5 and Fig. 2). In pineapple, some spots of proteins disappeared and some became bigger and clearer with increasing night temperature (Table 5 and Fig. 1). Former studies suggested that lipid constituents of biomembranes can be stabilized by the interaction with membrane proteins (Parola 1993). Thus, lipid-protein bilayer of pineapple and *K. pinnata* tonoplasts in response to high night temperature might be different. The difference might further influence on malate transport across the tonoplast. Behzadipour *et al*. (1998) reported that exposure of *K. daigremontiana* plants to high growth temperature induced a 35 kDa polypeptide in the tonoplast which cross-reacted with an antiserum against the tonoplast H^+-ATPase holoenzyme. With increasing night temperature, different increase rate of ATPase activity between pineapple and *K. pinnata* occurred. These findings spurred us to find the differences in ATPase for the two CAM species. The putative transporters of ATPase also showed obvious difference between pineapple and *K. pinnata* (Table 4). We inferred that the vacuole H^+-ATPase subunit B (Atlg20260.1 in Figs. 1 and 2) might be the 35 kDa protein, which was found by Behzadipour *et al*. (1998). This protein in the 2-D PAGE of pineapple could not be distinguished clearly. However, under the high night temperature, the spot became bigger and clearer compared with normal night temperature in *K. pinnata*. It is supposed that night temperature change also might affect the quantity and quality of tonoplast proteins (Fig. 1 and 2).

In summary, pineapple tonoplasts compared with the *K. pinnata* could keep higher rigidity under high night temperatures. Also, tonoplast ATPase of pineapple might provide more driving force for malate influx into the vacuole than that of *K. pinnata* under high night temperature. Thus, the difference of nocturnal malate accumulation between the two CAM species under high night temperature can be explained in this way. Analysis of tonoplast protein through 2-D PAGE showed different protein distribution and composition between pineapple and *K. pinnata*. This may be also the reason why pineapple could keep some levels of malate accumulation in the vacuole under the high night temperature, whereas *K. pinnata* could not.

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