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Tonoplast Lipid Composition and Proton Pump of Pineapple Fruit During Low-Temperature Storage and Blackheart Development

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Abstract Vacuole represents a major storage organelle playing vital roles in pH homoeostasis and cellular detoxification. The chemical and functional properties of tonoplast in response to chilling temperature and their roles in chilling injury are largely unknown. In the current study, lipid composition of tonoplast and the activities of two vacuolar proton pumps, H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (V-PPase), were investigated in accordance with the development of blackheart, a form of chilling injury in pineapple fruit (*Ananas comosus*). Chilling temperature at 10 °C for 1 week induced irreversible blackheart injury in concurrence with a substantial decrease in V-ATPase activity. By contrast, the activity was increased after 1 week at 25 °C. The activity of V-PPase was not changed under both temperatures. Level of total phospholipids of tonoplast decreased at 10 °C, but increased at 25 °C. There was no change at the level of total glycolipids under both temperatures. Thus, low temperature increased the ratio of total glycolipids vs. total phospholipids of tonoplast. Phosphatidylcholine and phosphatidylethanolamine were the predominant phospholipids of tonoplast. Low temperature increased the relative level of phosphatidic acid but decreased the percentage of both phosphatidylcholine and phosphatidylethanolamine. Unsaturated fatty acids accounted for over 60 % of the total tonoplast fatty acids, with C18:1 and C18:2 being predominant. Low temperature significantly decreased the percentage of C18:3. Modification of membrane lipid composition and its effect on the functional property of tonoplast at low temperature were discussed in correlation with their roles in the development of chilling injury in pineapple fruit.

Keywords Tonoplast · Vacuolar H^+-ATPase · Vacuolar H^+-pyrophosphatase · Lipid composition · Chilling injury · Pineapple fruit

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Introduction

Low temperature is one of the most important factors affecting plant growth and geographical distribution (Theocharis et al. 2012). Plants adapted to tropical and subtropical climates are often susceptible to chilling injury. Blackheart (also called internal browning) is a chilling injury developed in pineapple fruit (*Ananas comosus*) following exposure to low temperature, either in field or in postharvest storage (Teisson et al. 1979; Smith 1983; Paull and Rohrbach 1985). Most cultivars of pineapple develop blackheart symptoms during storage at <15 °C or after removal to room temperature (20–25 °C) from chilling condition (Teisson et al. 1979; Smith 1983; Paull and Rohrbach 1985). Blackheart has caused significant loss in pineapple production countries. The development of the injury involved the oxidation of phenolic substrates by polyphenol oxidase (PPO), which resulted in the formation of quinone products and brown polymers (Teisson et al. 1979; Stewart et al. 2001; Zhou et al. 2003a). Upregulation of PPO gene and enzyme, together with high level of phenolics, was reported in pineapple fruit following low-temperature storage

(Stewart et al. 2001; Zhou et al. 2003b; Youryon et al. 2007; Imbault et al. 2011). In living tissues, the PPO enzyme and its phenolic compounds are separated, with most PPOs being plastid-localized and their phenolic substrates accumulating in the vacuole (Mayer 1987). Chilling-induced damage of intracellular membranes, leading to loss of cell compartmentation is apparently a prerequisite for the contact of the enzyme with its substrate (Mayer 1987). Low temperature was recently shown to induce lipid modification of plasma membrane, which was in correlation with the loss of the functional properties of the plasma membrane during blackheart development in pineapple fruit (Zhou et al. 2014). Question remains as to what extent of the chilling induced changes in the endomembrane system, and how these changes lead to loss of sub-cellular compartmentation of the PPO enzyme and its phenolic substrates, leading to the subsequent browning reaction.

In mature cells, vacuole represents a major storage organelle for metabolites and nutrients. Vacuolar import and export not only balance the concentration of solutes in cytosol in accordance with cellular demands, but also fulfil active detoxification by acting as a repository for toxic compounds (Schulze et al. 2012). In this context, tonoplast functions in sustaining the sub-cellular compartmentation of phenolic compounds, maintaining its separation from PPO enzymes (Mayer 1987; Schulze et al. 2012). Two vacuolar proton pumps, H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (V-PPase), participate in establishing a proton motive force (pmf) across tonoplast to drive secondary transport of sucrose, organic acids and secondary metabolites into vacuole (Maeshima 2000; Dietz et al. 2001). The cold inactivation of V-ATPase has been demonstrated in chilling-sensitive species, such as rice (Kasamo 1988a) and mung bean (Kasamo 1988b; Yoshida et al. 1989; Yoshida and Matsuura-Endo 1991). Increasing evidence indicates that sustaining vacuolar H^+-ATPase activity under low temperature is involved in cold acclimation (Orr et al. 1995; Schulze et al. 2012). On the other hand, increase in V-PPase gene transcript and enzyme in seedlings of rice and mung bean under chilling was proposed as a backup system for V-ATPase under energy stress (Darley et al. 1995; Maeshima 2000). Nevertheless, the functional contribution of the two vacuolar proton pumps in response to chilling in fruit tissues of many species remains to be determined.

Lipid composition of endomembrane plays an important role in the regulation of both V-ATPase and V-PPase activities (Nakamura et al. 1997; Yoshida et al. 1999; Kasamo et al. 2000). Its role in determining the physical and functional properties of cell membranes at low temperature is well known (Lyons 1973; Nishida and Murata 1996). However, responses of tonoplast lipid composition to low temperature were only reported on a couple of species, including a crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana* grown at cooler temperature (Behzadipour et al. 1998). The chilling response of tonoplast lipid components and its role in chilling sensitivity are largely unknown. Analysis of the chemical and functional properties of tonoplast during the development of chilling injury could gain insights into the physiological role of tonoplast in plant tolerance to chilling temperature.

In the current study, for the first time, the lipid composition of tonoplast in response to chilling temperature was characterised together with the activity of two vacuole proton pumps, V-ATPase and V-PPase. The functional property of tonoplast in relation to the change of the chemical composition of the membrane under low temperature was discussed in correlation with its role in the development of chilling injury in pineapple fruit.

Material and Methods

Plant Material

Mature pineapple fruits (*Ananas comosus*, Smooth Cayenne) were harvested at a local farm outskirts of Guangzhou, China at summer season (July–September). Batches of 50 fruits were stored at 10 °C for 1 week and then transferred to 25 °C for 3 more weeks. A further 50 fruits were stored at 25 °C for 4 weeks as a control. All fruits were held at 95 % humidity. The experiments were replicated three times. Determination of blackheart injury was carried out weekly. Preparation of tonoplast vesicles, activity assay for V-ATPase and V-PPase, and lipid analysis were carried out at harvest and one week later. At least 10 samples for each treatment were used for blackheart assessment and three samples for tonoplast isolation.

Preparation of Tonoplast Vesicles

Tonoplast membrane vesicles were prepared at 4 °C according to the procedure of Kasamo (1990) with minor modification. Fruit pulp tissues (~120 g) were homogenized in ice-cold medium (1:3 w/v) [0.1 M Tris-Mes pH 7.7 with 4 mM dithiothreitol (DTT), 10 mM ethylene diamine tetraacetic acid (EDTA), 10 mM ethylene glycol bis(2-aminoethyl)tetraacetic acid (EGTA), 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 % (w/v) BSA and 2 % (w/v) soluble polyvinylpolypyrrolidone (PVP)]. The homogenate was filtered through serval layers of cheesecloth and centrifuged at 13,000×g for 15 min. The supernatant was then centrifuged for 30 min at 80,000×g to obtain microsomal pellet. The pellet, resuspended in 5 mL of suspension medium [10 mM on Tris-Mes pH 7.3, 0.25 mM sucrose, 1 mM DTT, 0.2 mM PMSF and 1 mM EGTA], was loaded on a step gradient

containing 6% dextran T-70 (w/v) in the suspension buffer. Following centrifugation at 100,000×g for 2 h, the turbid band, visible at the interface of 0/6 % dextran, was collected, diluted in the suspension buffer and pelleted at 100,000×*g* for 60 min. The resulting pellets were immediately extracted for lipid analysis, or resuspended in medium [5 mM Tris-Mes pH 7.3, 0.25 M sucrose, 1 mM EGTA and 1 mM DTT] for immediate enzyme assay.

The purity of tonoplast fraction was verified using marker enzymes defined for different sub-cellular membranes (Ferrol and Bennett 1996). The nitrate-sensitive ATPase, vanadate-sensitive ATPase and azide-sensitive ATPase were used as tonoplast, plasma membrane and mitochondrial membrane markers. Antimycin-insensitive NADH-cytochrome c reductase and latent inosine diphosphatase (latent IDPase) were used for the endoplasmic reticulum (ER) and Golgi apparatus markers.

Enzyme Assay

The V-PPase and V-ATPase hydrolytic activities were measured as the liberation of the inorganic phosphate released from ATP or PPi as described by Ames (1966). ATPase activity was measured at 30 °C for 30 min according to Shiratake et al (1997). The reaction was initiated by the addition of membrane suspension (10 ∼ 20 μg of membrane protein) to a reaction mixture [50 mM Mes-BTP pH 7.5, 3 mM Tris-ATP, 50 mM KCl, 3 mM MgSO4 and 0.02 % (w/v) Triton X-100]. The reaction mixture also contained 0.25 mM ammonium molybdate to inhibit acid phosphatase. The amount of Pi released was colorimetrically determined as previously described (Ames 1966). The activity was expressed as nmol Pi/min/mgProtein. For inhibition analysis, the reaction was measured in the presence of 50 mM KNO_3 (pH 7.5), 0.1 mM Na_3VO_4 (pH 7.5) or 1 mM NaN_3 (pH 8.0), respectively. The activity of tonoplast V-ATPase (nitrate-sensitive ATPase) was calculated from the difference between the absence and presence of KNO_3. The activity of V-PPase activity was measured at 30 °C for 30 min (Shiratake et al. 1997). The reaction mixture contained 50 mM BTP-Mes pH 7.5, 50 mM KCl, 1 mM MgSO4, 0.02 % (w/v) Triton X-100, 1 mM sodium pyrophosphate and 0.25 mM ammonium molybdate. The enzymatic activities of V-PPase were colorimetrically measured by the amount of Pi release as above. Activity of antimycin-insensitive NADH-cytochrome c reductase was determined as described by Hodges and Leonard (1974). Activity of latent IDPase was assayed according to Pinton *et al* (1994). Protein concentration was determined according to Bradford (1976).

Lipid Analysis

Membrane lipid was extracted from tonoplast membrane according to the procedure of Zhou *et al* (2014). Essentially, the tonoplast pellet (above) was first mixed with boiling isopropanol, for 5 min, then extracted with chloroform:methanol (2:1 v/v) containing butylhydroxytoluol (50 μg/mL). The chloroform phase was recovered and concentrated to a smaller volume under a stream of N_2. The polar lipid fraction was separated from the total lipid extract by thin-layer chromatography (TLC) on activated silica gels with hexane:diethyl ether:acetic acid (70:30:1 v/v/v) as mobile phase. Lipid classes were identified by co-chromatography with standard chemicals and visualized with iodine vapour. The polar lipid fractions were scraped off plates and quantitated for phosphorus (Rouser et al. 1970) and glucose contents (Roughan and Batta 1968). The polar lipid fraction was further separated into individual phospholipid species by two-dimensional TLC using chloroform–methanol–NH_3–water (68:27:4:1) followed by chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5). Identity of individual phospholipids, phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidic acid (PA) was confirmed by co-chromatography with known standards, lipid spots were visualized and quantitated by their phosphorus content as above.

For Fatty acids analysis, the total fatty acids of membrane lipid extract were saponified and methylated with BF3 as previously described (Zhou et al. 2014). Heptadecanoic acid (C17:0) was added as an internal standard. Fatty acid methyl esters were analysed by GC (Shimadzu GC-9A) equipped with a BD-5 capillary column (30 m × 0.25 mm × 0.25 um, Shimadzu Scientific). The oven temperature was programmed from 120 °C to 260 °C at 4 °C/min. The injector and detector temperatures were 200 °C and 250 °C, respectively. Fatty acid methyl esters were identified by comparing the retention times with known standards, peak areas were calculated by the equipped automatic data analysis system and presented as relative percentage of the total components. All chemical reagents and standards were purchased from Sigma Aldrich Co. Ltd.

Measurement of Blackheart Symptom, Chilling Injury of Pineapple Fruit

For blackheart assessment, a total of ten fruits of each treatment were halved longitudinally, the incidence and severity of blackheart injury were visually scored from 0 (no blackheart) to 6 (maximum blackheart) according to Zhou et al (2003a).

Statistical Analysis

Significant differences were tested using analysis of variance (ANOVA) followed by Tukey's multiple comparison test at $P < 0.05$.

Results

Blackheart Development During Low-Temperature Storage

Most cultivars of pineapple develop blackheart symptom during storage at <15 °C or after removal to room temperature (20–25 °C) from chilling condition (Teisson et al. 1979; Smith 1983; Paull and Rohrbach 1985). The first sign of blackheart symptom is a small brown translucent spots forming at the base of fruitlets near the core (Smith 1983; Paull and Rohrbach 1985). This early symptom could be detected in pineapple fruits from one to seven days after removal from 10 °C temperature to ambient temperature (25 °C) (Fig. 1). The symptom rapidly progressed, with the severity of damage increasing following removal from chilling to 25 °C (Fig. 1). By contrast, blackheart was not observed in pineapple fruit continuously stored at 25 °C for 4 weeks (Fig. 1). The results supported previous findings that blackheart was closely related to low-temperature treatment, and indicated that the treatment of 10 °C for one week caused irreversible chilling injury in pineapple fruit.

Effect of Low Temperature on V-ATPase and V-PPase Activities of Tonoplast

The purity of the tonoplast fractions was assessed by the activities of marker enzymes, nitrate-, vanadate- and azide-sensitive ATPase, NADH-cytochrome c reductase and Latent IDPase. The ATPase activity of the tonoplast fractions was strongly inhibited by KNO_3, but insensitive to azide across all the samples (Table 1). The contamination of plasma membrane was low, with average 11 % inhibition of total ATPase activity by vanadate. The level of NADH cyt c reductase and latent IDPase activities, representing on average 8.9 and 8.3 % of the total ATPase activity, respectively (Table 1), was slightly lower that those previously reported, about 10 % of total ATPase (Dupont 1987; Etxeberria and Gonzalez 2004), indicating small amount of ER and Golgi contamination. Overall, these results indicated that the membrane fractions used in the current study were enriched in tonoplast vesicles without significant contamination of other cellular membranes.

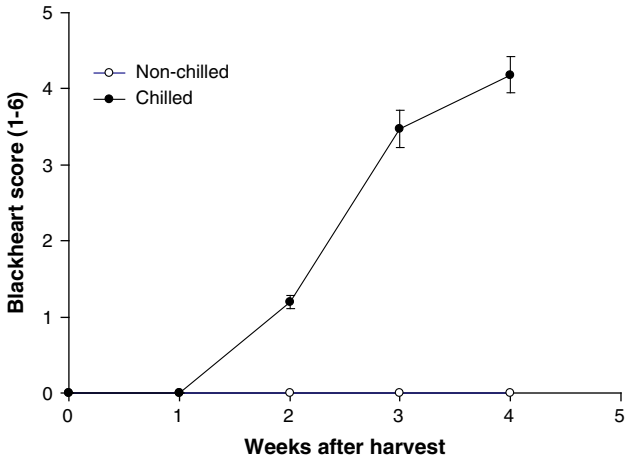

Fig. 1 Effect of low temperature on blackheart development of pineapple fruit. Non-chilled, continuous 25 °C; Chilled, 1 week storage at 10 °C followed by 3 weeks at 25 °C. The severity of blackheart injury was visually scored from 0 (no *blackheart*) to 6 (maximum *blackheart*). All values represent mean ± SE of ten biological replicates

The activity of tonoplast V-ATPase (KNO_3-sensitive ATPase) was substantially decreased by low temperature at 10 °C after 1 week with 33.5 % reduction compared with that at harvest, and 42.6 % reduction compared with that at 25 °C after 1 week (Fig. 2). By contrast, an increase of 15.8 % in ATPase activity was observed during storage at 25 °C for 1 week (Fig. 2). The activity of V-PPase was markedly lower than that of V-ATPase across all samples, and was not significantly changed during storage at either temperature (Fig. 2). The ratio of V-ATPase activity vs. V-PPase activity was 3.9, 4.4 and 2.4 for fruit at harvest, 1 week storage at 25 and 10 °C, respectively (Fig. 2).

Effect of Low Temperature on Lipid Composition of Tonoplast

Low temperature for 1 week was shown to significantly decrease the level of total phospholipids, with 34.5 % reduction compared with that at harvest and 45.8 % reduction compared with that at 25 °C for 1 week (Fig. 3). By contrast, the total phospholipid content was increased by 21 % after 1 week at 25 °C. The level of total glycolipids was not changed under either temperature (Fig. 3). Therefore, a significant increase in ratio of total glycolipids vs. total phospholipids compared with that at harvest was shown following low-temperature storage (0.61 vs. 0.38). By contrast, the ratio was not significantly changed during 25 °C storage (0. 34 vs. 0.38) (Fig. 3). The phospholipid species of tonoplast isolated from pineapple fruit included phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA) (Fig. 4). The PC and PE were the predominant phospholipid species, together accounting for over 70 % of the total phospholipids in tonoplast of harvested fruit. The PG (~2 %) was in the least amount. The ratio of PC/PE was ~2:1 (Fig. 4). Significant effects of low temperature on phospholipid composition were the increase of PA, with 91.5 % higher than that at harvest, and the decrease of both PC and PE, being 20 and 22 % lower, respectively (Fig. 4). However, these changes did not lead to significant alteration of the PC/PE ratio (1.97 vs. 2.04 at harvest). For the

Table 1 Activities of marker enzymes in tonoplast fractions isolated from pineapple fruit

Marker enzymes	Harvest	25 °C 1 week	10 °C 1 week
ATPase (no addition, control)	735.44 ± 38.08	855.56 ± 40.36	483.66 ± 22.59
% of control			
No addition	100	100	100
+50 mM KNO_3	21.75 ± 1.40	22.10 ± 1.42	20.91 ± 1.25
+0.1 mM Na_3VO_4	89.77 ± 4.78	88.52 ± 4.62	88.03 ± 4.24
+NaN_3	101.03 ± 4.28	99.31 ± 4.01	102.40 ± 4.34
NADH-cyt c reductase	61.78 ± 2.87	77.51 ± 3.26	45.19 ± 1.86
Latent IDPase	58.39 ± 2.79	71.52 ± 3.07	41.45 ± 1.68

Activities of ATPase and latent IDPase are presented as nmol Pi/min/mgProtein, the NADH cyt c reductase activity is presented as nmol cyt/min/mgProtein

All values represent the mean ± SE of six biological replicates

Values with different letters are significantly different ($P < 0.05$)

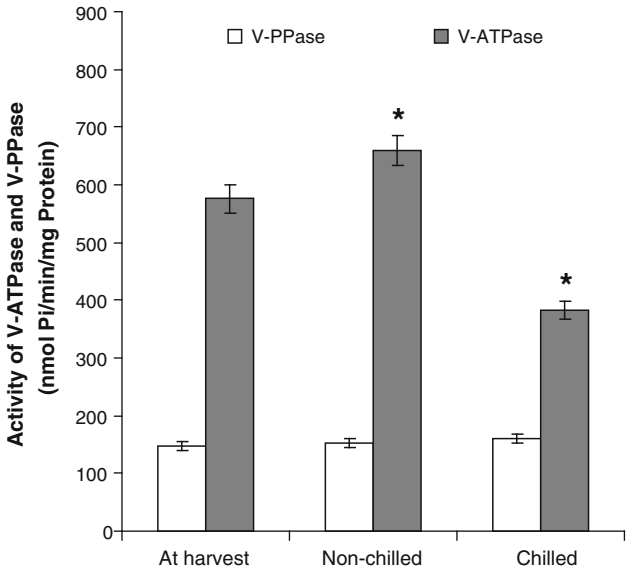

Fig. 2 Effect of low temperature on the activity of vacuolar H^+-ATPase and H^+ pyrophosphatase of pineapple fruit. Non-chilled, 25 °C 1 week; Chilled, 10 °C 1 week; V-ATPase, vacuolar H^+-ATPase; V-PPase, vacuolar H^+ pyrophosphatase; All values represent mean ± SE of six biological replicates (*$P < 0.05$)

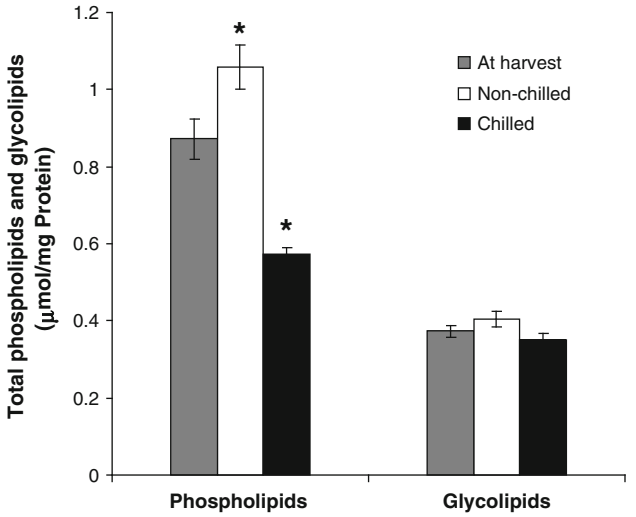

Fig. 3 Effect of low temperature on total phospholipids and glycolipids of tonoplast isolated from pineapple fruit. Non-chilled, 25 °C 1 week; Chilled, 10 °C 1 week. All values represent mean ± SE of six biological replicates (*$P < 0.05$)

rest of the phospholipid species, the difference between treatments was not significant. There was no significant change in the phospholipid profile after storage at 25 °C for 1 week (Fig. 4).

The detectable fatty acids of tonoplast were myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) (Table 2). The unsaturated fatty acids accounted for over 60 % of the total tonoplast fatty acids of harvested fruit, with C18:1 and C18:2 being predominant followed by C18:3 (Table 2). The relative level of most fatty acid species was not changed after one week at either temperature, except that of C18:3 where significant decrease from 12.02 to 8.90 % was shown under low-temperature storage. However, the change of DBI value was not significant (Table 2).

Discussion

Treatment of 10 °C for one week caused irreversible chilling injury in pineapple fruit although visible symptoms started after removal from 10 to 25 °C (Fig. 1). The result that V-ATPase activity was decreased after 1 week of chilling (Fig. 2) agreed with previous reports in seedlings of rice and mung bean (Kasamo 1988b; Yoshida et al. 1989; Yoshida and Matsuura-Endo 1991). Structural disintegration has been shown to account for the chilling-

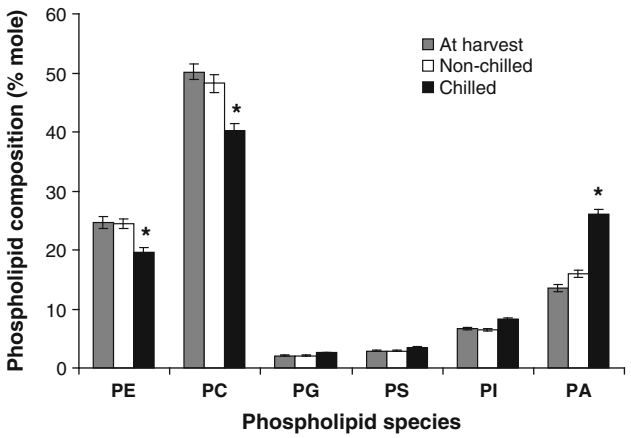

Fig. 4 Effect of low temperature on phospholipid composition of tonoplast isolated from pineapple fruit. Non-chilled, 25 °C 1 week; Chilled, 10 °C 1 week. *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PI* phosphatidylinositol, *PS* phosphatidylserine and *PA* phosphatidic acid; All values represent mean ± SE of six biological replicates (*$P < 0.05$)

induced inactivation of V-ATPase in mung bean hypocotyls (Matsuura-Endo et al. 1990). It was found that the stability of V-ATPase to cold was distinctly different between cold-sensitive and insensitive species (Yoshida et al. 1999). Sustaining V-ATPase activity was correlated with cold-induced solute accumulation in the vacuole and clod acclimation (Schulze et al. 2012). Furthermore, it was demonstrated that cold-induced inactivation of V-ATPase was closely linked to the rapid acidification of the cytoplasm and, therefore, the major cause of irreversible injury (Yoshida 1994; Yoshida et al. 1999). Therefore, chilling-induced inhibition of V-ATPase in pineapple fruit may reflect one of the primary cellular events from exposure to low temperature.

The significantly higher activity of V-ATPase compared to that of V-PPase in pineapple fruit, together with the results that activity of V-ATPase, not V-PPase, increased following ripening (Fig. 2) supported the critical role of ATPase to meet the increasing demand during ripening process. Previous reports indicated that fluxes to vacuole increased during fruit ripening (Moing et al. 2001; Borsani et al. 2009; Yin et al. 2010). Increases in organic acid content, such as citric and malic acid, and accumulation of phenolic compounds have been reported in ripening pineapple fruit (Zhou and Tan 1997; Dahler et al. 2000; Weerahewa and Adikaram 2005; Saradhuldhat and Paull 2007; Nilprapruck et al. 2008). In fact, a mature pineapple fruit can have a juice pH down to 3.1 during ripening (Paull and Chen 2003). Therefore, assuming a cytosolic pH of 7.2, the trans-membrane ΔpH across tonoplast may correspond to ~4 units. Consequently, even under normal condition the tendency of the permeable H^+ ions to flow down their concentration gradient should be balanced according to metabolic demand through continuous regulation of vacuolar proton pumps. Our results suggested that in comparison with V-PPase, V-ATPase may provide a greater driving force for the secondary transport of metabolites and nutrients into vacuole during pineapple ripening.

It has also been suggested that typical V-ATPases operate far from thermodynamic equilibrium (Müller et al. 1999), the contribution of V-PPase to pH homoeostasis may be critical. The importance of V-PPase under environmental stresses was evidenced by previous reports that under anoxia and reversible cold stress, a transient substantial increase of V-PPase was observed in anoxia-tolerant species, which may function to replace V-ATPase under energy stress to maintain vacuole acidity (Darley et al. 1995; Maeshima 2000). In contrast to the reversible phase of chilling injury, our result that V-PPase activity was not significantly increased after 1 week at 10 °C (Fig. 2) was consistent with previous report on the hypocotyls of mung bean suffering irreversible chilling-induced damage (Yoshida et al. 1989; Kawamura 2007) and suggested a sign of irreversible injuries at vacuole of pineapple fruit induced by 10 °C treatment.

Table 2 Effect of low temperature on fatty acid composition (mol%) of tonoplast isolated from pineapple fruit

Fatty acids	At harvest	1 week 25 °C	1 week 10 °C
Myristic (C14:0)	1.35 ± 0.07a	1.32 ± 0.06a	1.28 ± 0.06a
Palmitic (C16:0)	29.89 ± 1.35a	30.65 ± 1.24a	32.16 ± 1.52a
Palmitoleic (C16:1)	0.69 ± 0.03a	0.78 ± 0.03a	0.61 ± 0.03a
Stearic (C18:0)	4.10 ± 0.18a	3.91 ± 0.19a	4.18 ± 0.18a
Oleic (C18:1)	26.24 ± 1.22a	26.84 ± 1.11a	24.86 ± 1.17a
Linoleic (C18:2)	25.69 ± 1.11a	25.43 ± 1.06a	28.04 ± 1.43a
Linolenic (C18:3)	12.02 ± 0.49a	10.07 ± 0.45a	8.90 ± 0.35b
DBI	1.14 ± 0.06a	1.12 ± 0.04a	1.08 ± 0.04a

DBI double bound index = Σ (unsaturated fatty acid × number of double bound)/100

All values represent the mean ± SE of six biological replicates

Values with different letters from the same row are significantly different ($P < 0.05$)

Chilling temperature has been reported to induce accumulation of acids and soluble sugar in many plants, including pineapple fruit (Wormit et al. 2006; Hu et al. 2011; Theocharis et al. 2012; Hong et al. 2013). Low temperature also upregulated the activity of phenylalanine ammonia-lyase (PAL), a key enzyme for phenolic biosynthesis in pineapple fruit (Zhou et al. 2003a). Indeed, decrease in juice pH from 3.5 to 2.8 has been reported in pineapple fruit under cold storage (Paull and Chen 2003). However, the ability to sustain compartmentation of these solutes within the vacuoles must depend on the transport system across the tonoplast (Wormit et al. 2006). Two vacuolar proton pumps, V-ATPase and V-PPase, energize the massive fluxes of ions and metabolites that are not only required for nutrient storage but also for detoxification (Maeshima 2000; Dietz et al. 2001). Our results that the reduced V-ATPase activity was not compensated by any increase of V-PPase activity under chilling in pineapple fruit would predict that the vacuole would be incapable of generating sufficient *pmf* across tonoplast to support the cold-induced active transport of ions and metabolites. These may lead to impaired vacuolar sequestration of toxic solutes, such as phenolic compounds. Indeed, vacuolar membrane collapse and cytosolic pH decrease were recently demonstrated as an initial phase of leaf damage induced by low temperature (Kadohama et al. 2013).

Tonoplast V-ATPase is known to require phospholipids for its activity (Kasamo 1988a; Matsuura-Endo et al. 1990; Yamaguchi and Kasamo 1993). The substantial loss in total phospholipids of tonoplast at low temperature (Fig. 3) may explain the concurrent decrease in activity of V-ATPase (Fig. 2). While the absolute quantity of total glycolipids was not changed after low-temperature storage, the ratio of total glycolipid vs. phospholipids was significantly increased from 0.38 to 0.61. Glycolipids are considered as non-lamellar or hexagonal phase lipids, their roles have been suggested to provide efficient sealing of membrane proteins to the bilayer (Leach et al. 1990). Kasamo et al (2000) demonstrated that the activity of V-ATPase was suppressed by glycolipids of tonoplast in chilling-sensitive rice. The tonoplast glycolipids also decreased the phospholipid-induced activation of V-ATPase (Yamaguchi and Kasamo 2001). In contrast, the progressive decrease in the proportion of glycolipids leading to changes in cryostability of the plasma membrane was correlated to cold acclimation in rye and oat membranes (Steponkus and Lynch 1989; Uemura and Steponkus 1994).

Increase in phosphatidic acid (PA) accompanying with decrease in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of tonoplast at low temperature (Fig. 4) suggested accumulation of PA through degradation of tonoplast membrane phospholipids, such as PC and PE, may occur under low-temperature treatment. Phospholipid hydrolysis by phospholipase D (PLD) has been suggested as a pathway leading to the loss in membrane phospholipids and formation of PA (Bargmann and Munnik 2006; Testerink and Munnik 2011). As a result, high content of PA in tonoplast can have profound effect on membrane architecture and membrane-bound proteins due to its small polar head-group and two fatty acyl side chains (Kooijman et al. 2003; Testerink and Munnik 2011). Accumulation of PA in membranes has been shown to promote formation of hexagonal II-phase lipid particles with increase in membrane permeability (Berglund et al. 2001) and loss of membrane integrity (Kooijman et al. 2003; Roth 2008). During wounding or senescence, high content of PA induced by phospholipase D was found to destabilize membranes, leading to loss of both membrane integrity and functionality of membrane proteins (Fan et al. 1997; Thompson et al. 1998). The freezing-induced high content of PA was also associated with membrane damage and cell death (Welti et al. 2002; Devaiah et al. 2007). By contrast, suppression of PA was reported to decrease the formation of non-bilayer phase and membrane ion leakage during freezing (Welti et al. 2002). A much lower PA in *T. salsuginea* compared with *A. thaliana* during freezing was responsible for a less membrane injury in the former (Welti et al. 2002; Zhang et al. 2013). Collectively, these results suggested the low temperature-induced increase in phosphatidic acid may destabilize tonoplast and lead to loss of membrane integrity. These, together with impaired proton pump activity, may result in loss of vacuolar compartmentation of phenolic substrates. In fact, increase of membrane leakage was detected at the same time (Zhou et al. 2014). These events were accompanied by a parallel induction of the transcription of PPO genes by low temperature while the *de novo* synthesis of the PPO enzyme only occurred at subsequent incubation at ambient temperature (Stewart et al. 2002; Zhou et al. 2003b). The oxidation of phenolic compounds by PPO produces *o*-quinones which can undergo polymerisation and bind covalently to nucleophilic amino acids to form black or brown pigments leading to the characteristic browning of fruit and vegetables (Mayer 1987). It has also been predicted that damage of endomembrane system may have contributed to the disorganization of internal thylakoid membranes which leads to the release of the plastid-bound PPO enzyme and the subsequent contact with its phenolic substrates (Mayer 1987), the mechanism of this process remains to be characterised.

Apart from being a key membrane component, PA is a typical signalling lipid with diverse downstream responses. Accumulation of PA through activation of PLD plays critical role in regulating plant response to H_2O_2, and ABA (Zhang et al. 2003; Testerink and Munnik 2011). PA was also demonstrated to stimulate ROS production (Zhang et al.

2009). There is evidence to suggest H_2O_2 and balance of plant hormone abscisic acid (ABA) and gibberellins may be involved in the development of blackheart (Zhou and Tan 1992; Zhou and Tan 1997; Zhou et al. 2003b; Nukulthornprakit and Siriphanich 2005; Pusittigul et al. 2012). In this context, the signalling role of PA in mediating chilling injury in pineapple fruit deserves further investigation.

While cooler growth temperatures are often associated with increase in the production of polyunsaturated fatty acids (PUFAs) in many species, reports on fatty acid unsaturation of tonoplast in response to low temperature are not plentiful. A report indicated that phenotypic adaptation of tonoplast occurred in a CAM plant *Kalanchoë daigremontiana* with increase in tonoplast fluidity and percentage of C18:3 following exposure to low temperature (Behzadipour et al. 1998). Low temperature-induced increase in unsaturated fatty acids of tonoplast was also observed at cultured cells of rice with non-fatal cell damage (Kasamo et al. 1992) and hypocotyls of *Vigna unguiculata* during the reversible phase of chilling injury (deOliveira et al. 2009). These works were commonly discussed in term of acclimation rather than chilling injury (Kasamo et al. 1992; Behzadipour et al. 1998). In contrast, our results that significant reduction in the relative level of C18:3 was shown in tonoplast after chilling (Table 2) reflected an irreversible phase of chilling injury where visible blackheart symptoms developed following removal from chilling condition (Fig. 1). The change pattern was in parallel with our recent findings that decrease in unsaturated fatty acids together with increase in membrane permeability occurred in plasma membrane of pineapple fruit under low-temperature treatment, and in agreement with membrane damage reported on *Solanum tuberosum*, cucumber, green bell pepper and peach during the development of chilling injury (Parkin and Kuo 1989; Lurie et al. 1994; Wismer et al. 1998; Zhang and Tian 2010). Decrease in percentage of linolenic acid of plasma membrane was correlated with lower membrane fluidity in peach fruit during the development of chilling injury at 5 °C, whereas maintenance of high linolenic acid accumulation was correlated to greater membrane fluidity and chilling tolerance (Zhang and Tian 2009; Zhang and Tian 2010). Transgenic tomato plant, a chilling-sensitive species accumulating higher level of C18:3 through overexpressing omega-3 fatty acid desaturase genes, was reported to reduce the chilling-induced increase of electrolyte leakage and enhanced tolerance to chilling temperature (Yu et al. 2009; Domínguez et al. 2010; Liu et al. 2013). Finally, decrease in PUFAs of tonoplast was correlated with lower activities of V-ATPase and V-PPase in Arabidopsis *fad2* mutant deficient in a microsomal ω-6 fatty acid desaturases (Zhang et al. 2012). Collectively, our results demonstrated that decrease in PUFAs of tonoplast preceded the development of irreversible blackheart injury in pineapple fruit under low-temperature storage. These may lead to decrease in tonoplast membrane fluidity and, therefore, impairment of membrane-bound proteins such as V-ATPase.

Modification in the lipid composition of tonoplast can be caused by lipid peroxidation due to ROS and/or LOX (Berglund et al. 2001). Previous reports that during blackheart development level of H2O2 and LOX activity increased together with decrease in cell protective system, such as activity of antioxidant enzymes (Zhou et al. 2003a; Nukulthornprakit and Siriphanich 2005; Youryon et al. 2007), would support a role of lipid peroxidation in the degradation of tonoplast phospholipids under chilling. On the other hand, linolenic acid, as a substrate for oxylipin pathway by LOX, is connected to diverse cellular functions including the biosynthesis of jasmonic acid, a signalling molecule associated with wounding response (Porta and Rocha-Sosa 2002). Noticeably, the transcription of pineapple PPO gene was wound-inducible (Stewart et al. 2002; Zhou et al. 2003b). It would be interesting to establish whether the lipid degradation of tonoplast could be connected with the induction of PPO under chilling.

Taken together, our results provided first evidence that changes of tonoplast lipid composition occurred in pineapple fruit in response to low-temperature storage at 10 °C. Loss of phospholipids, with increase in ratio of glycolipids to phospholipids and the level of phosphatidic acid, decrease in level of phosphatidylcholine and phosphatidylethanolamine, and decrease in percentage of C18:3 fatty acid of tonoplast, was correlated with the impaired membrane function as evidenced by the substantial decrease in V-ATPase activity; therefore, irreversible chilling injury developed in pineapple fruit under low-temperature storage.

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