Calcium-Stimulated Protein Kinase Activity of the Hypodermal-Mesocarp Plasma Membrane from Preharvest-Mature and Postharvest Muskmelon

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Vanadate-sensitive H^+-ATPase and calcium-dependent protein kinase (kinase) activities of hypodermal-mesocarp plasma membrane (PM) vesicles isolated from preharvest mature, harvested, and stored muskmelons (*Cucumis melo* L. var. reticulatus Naud.) decreased following harvest. Kinase activity is markedly stimulated by Ca^{2+} and is responsible for phosphorylation of many melon PM proteins. Specifically, a PM protein band at 97 kDa, immunodetected as H^+-ATPase, appeared phosphorylated in mature fruits, but phosphorylation and the sodium dodecyl sulfate (SDS) gel protein band were not detected in PM from harvested or stored fruit. Kinase peptide, immunodetected at 63 kDa, was present in all PM tissues, but kinase phosphorylation activity decreased with fruit harvest and storage. However, PM kinase activity in the presence of exogenous Ca^{2+} and histone III−S was greatly increased in harvested, and stored fruit, indicating both a stimulatory effect of Ca^{2+} and a loss of a suitable endogenous kinase substrate in postharvest melons. Washing PM vesicles with EGTA to remove Ca^{2+} and Ca^{2+} from Ca^{2+}-binding proteins, e.g. calmodulin, or adding brain calmodulin affected kinase activity only in preharvest mature melon tissue. Our data indicate that the loss in kinase phosphorylation activity after harvest in melon PM is most likely due to the decrease in H^+-ATPase peptide, a known kinase substrate, and that the decrease in H^+-ATPase activity in postharvest fruit PM is most likely due to a loss in peptide content rather than its phosphorylation status by kinase.

Keywords: *Cucurbitaceae; Cucumis melo; calcium dependent protein kinase; H^+-ATPase; fruit; plasma membrane*

INTRODUCTION

Calcium's role as a secondary messenger in the growth and senescence of plant tissues is well-documented (Ferguson and Drobak, 1988; Poovaiah, 1988; Leshem, 1992). One major investigative area of Ca^{2+} activity in plant growth and senescence is plasma membrane function and structure (Michelet and Boutry, 1995). The effect of Ca^{2+} on plasma membranes is attributed to stabilization and maintenance of integrity (Glenn et al., 1988; Lurie et al., 1987; Nur et al., 1986; Paliyath and Droillard, 1992) and regulating H^+-ATPase activity (Hanson and Trewavas, 1982). Ca^{2+} is beneficial in retarding membrane senescence cytoplasmically in the cell (Leshem, 1992). Calcium enters the cytoplasm via voltage regulated Ca^{2+}-permeable channels (Huang et al., 1994) and enters and exits via ATPase (Reid et al., 1995), and Ca^{2+}/H^+ antiporter channels (Kasai and Muto, 1990), located within the plasma membrane. Maintaining calcium homeostasis within the cytoplasm via active channels within the plasma membrane is extremely critical in maintaining cellular function (Felle et al., 1992; Roberts and Harmon, 1992), which is, therefore, critical in regulating whole plant tissue senescence.

In orange-fleshed, netted muskmelon fruit (*Cucumis melo* L. var. reticulatus Naud.) Ca^{2+} content in mesocarp tissue decreases nearly 2-fold following ripening and continues to decline with senescence (Wang et al., 1996). Concomitantly, hypodermal-mesocarp tissue plasma membrane H^+-ATPase activity decreases more than 2-fold following fruit ripening and senescence (Lester and Stein, 1993). Incubating hypodermal-mesocarp disks from ripe muskmelon fruit in low $CaCl_2$ concentrations retards plasma membrane senescence (Lester, 1996). Thus, calcium's effect in retarding muskmelon hypodermal-mesocarp plasma membrane senescence is directly related to maintenance of total phospholipids, proteins, and H^+-ATPase activity. Calcium can regulate plant senescence through activating calcium-dependent protein kinase (Heatherington et al., 1990; Polya and Chandra, 1990; Garbarino et al., 1991; Schaller and Sussman, 1988). This kinase, located near the surface on the cytoplasmic side of the plasma membrane, phosphorylates H^+-ATPase. During plant senescence

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Table 1. Vanadate-Sensitive H^+-ATPase Activity and Protein Content of Var. Cruiser Muskmelon Hypodermal-Mesocarp Plasma Membrane Tissue from Mature (2 Days prior to Abscission), Postharvest (30 h at 24 °C), and Storage (7 Days at 21 °C plus 90 ± 5% RH) Fruits

fruit tissue maturity	protein (mg·(g of fresh weight)$^{-1}$)	H^+-ATPase activity ((nmol of Pi)·min^{-1}·(mg of protein)$^{-1}$)	total H^+-ATPase activity ((nmol of Pi)·min^{-1}·(mg of total membrane protein)$^{-1}$)
mature	6.6 ± 0.5[a]	56 ± 9	371 ± 55
postharvest	5.6 ± 0.2	35 ± 4	194 ± 20
storage	5.5 ± 0.6	16 ± 2	88 ± 11

[a] Means $n = 3 \pm$ sd.

kinase phosphorylation normally decreases (Poovaiah, 1988). In apple *Malus sylvestris*, Mill (Paliyath and Poovaiah, 1985), and tomato *Lycopersicum esculentum* L. (Raghothama et al., 1985) fruits, kinase phosphorylation, assayed in a total microsomal membrane fraction, decreased with senescence. However, in tomatoes, exogenous free Ca^{2+} slightly promoted kinase phosphorylation in ripe fruit. It is unclear from these previous fruit senescence studies if heightened kinase activity due to exogenous Ca^{2+} application equates to heightened plasma membrane H^+-ATPase activity, resulting in extended fruit shelf life.

In the present muskmelon study our objective was to document calcium-dependent protein kinase (kinase) activity and to determine general phosphorylation in relation to declining plasma membrane H^+-ATPase activity following fruit maturity. The emphasis is on kinase activity of the hypodermal-mesocarp tissue plasma membrane proteins, because of this tissue's direct association with muskmelon fruit ripening and postharvest senescence (Lester and Stein, 1993). Knowledge from this study should provide direction for further research into programmed senescence of muskmelon fruit.

MATERIALS AND METHODS

Plant Material, Plasma Membrane Isolation, and H^+-ATPase Assay. Fruit of var. Cruiser muskmelon with a mean weight of 2.0 kg and free of defects were harvested just after sunrise from a commercial field located near Weslaco, TX. Fruits classified as "mature" were preclimateric and approximately 2 days prior to abscission, based on external characteristics (Lester and Dunlap, 1985). Fruits classified as "harvested" and "stored" had abscissed and were ripe and senescing, respectively. Fruits were washed in distilled water and either processed immediately (mature) or held in ventilated, waxed muskmelon shipping boxes for 24 h at 24 °C (harvested) or 7 days at 21 °C plus 90 ± 5% relative humidity (RH; stored).

Isolation of muskmelon hypodermal-mesocarp plasma membranes were by aqueous polymer two-phase (6.2% (w/w) dextran T500 and 6.2% (w/w) PEG 3400) partitioning followed by sucrose density (1.13−1.14 g·cm^{-1}) gradient purification, as described in Lester and Stein (1993). Protein content of the density fractions was determined by the method of Bradford (1976). Vanadate-sensitive H^+-ATPase (EC 3.6.1.8) activity of plasma membrane vesicles, with right-side out orientation (Kasai and Muto, 1990), was assayed at pH 6.5 and 38 °C, according to the modified method of Hodges et al. (1972). Reaction mixtures contained 1 mL of 3 mM Tris-ATP, 4 mM $MgSO_4$, 33 mM Tris-MES, 50 mM KNO_3, and 1 mM DTT with or without 1 mM Na_3VO_4. ATPase enzyme assays were stopped by adding 2 mL of 1.25% (w/v) ammonium molybdate in 2 N HCl. Inorganic phosphate was determined as a measure of H^+-ATPase activity by the method described by Peterson (1978).

Kinase Assays. Calcium-dependent protein kinase activity was assayed in 50 μL of 30 mM HEPES-Tris (pH 7.0), 5.5 mM $MgSO_4$, 1.5 mM EGTA (which keeps, in the absence of added $CaCl_2$, the free Ca^{2+} concentrated lower than 50 nM), 0.5 mM [γ-^{32}P]-ATP (3.7 TBq/mol) and, where indicated, $CaCl_2$ to have 20 μM free Ca^{2+} and 1 mg/mL histone III−S. The reaction was started by adding 2.5 μg of plasma membrane protein. After 5 min at room temperature, the reaction mixture was spotted onto 2.4 cm × 3.0 cm Whatman P81 phosphocellulose papers. These were washed with 75 mM H_3PO_4 (3 × 10 min) and rinsed in ethanol (1 × 5 min) and in diethyl ether (1 × 5 min). They were then air-dried, placed in vials with a scintillation liquid, and counted for radioactivity. The concentrations of free Ca^{2+}, free Mg^{2+} and Mg-ATP in assay media were calculated using the program Chelator (Schoenmakers et al., 1992).

Polyacrylamide Gel Electrophoresis. To observe phosphorylated proteins, 15 μg of plasma membrane proteins from hypodermal-mesocarp tissue of each fruit age were incubated in 5 μL of the same medium used to measure protein kinase activity, except that 0.05 mM [γ-^{32}P]-ATP (733 Tbq/mol) was used. The reaction was stopped after 6 min by adding 5 μL of 50 mM Tris-HCl (pH 6.8), 15% (v/v) glycerol, 5% (w/v) SDS, 2% (w/v) 2-mercaptoethanol, and 0.3% (w/v) bromphenol blue. Proteins were then separated by electrophoresis (Laemmli, 1970), and the gel was stained, as described by Schagger and Von Jagow (1987). The gel was then dried under vacuum and exposed to Dupont Cronex film.

Blotting and Immunodetection of H^+-ATPase and CDPK. Proteins (6 μg) from plasma membrane vesicle preparations were solubilized in Laemmli (1970) sample buffer at 37 °C and separated on a 12% SDS−polyacrylamide gel (SDS = sodium dodecyl sulfate) according to standard methods. The proteins were then electroblotted from the gels onto nitrocellulose membranes (MSI, Westborough, MA) using a semidry blotter (Sartorius, Hayward, CA) and a constant current of 1.2 mA/cm^2. Following electroblotting, the gels were stained with Coomassie blue to verify complete transfer of the proteins to the membranes. The plasma membrane H^+-ATPase was immunodetected with a chemiluminescent immunodetection kit from Tropix (Bedford, MA). The primary antibody was used at a concentration of 1/1000, while the secondary antibody concentration was 1/10 000. Polyclonal antibodies against *Elodea* which also recognized *Cucurbita* PM H^+-ATPase were prepared by W.M., and immunodetections were kindly performed by Dr. Marla Binzel (Texas A&M University, El Paso, TX). For calcium-dependent protein kinase detection, proteins were blotted onto nitrocellulose membranes in 25 mM Tris with 192 mM glycine (pH 8.0) for 120 min at 300 mA. Immunodetection for CDPK was kindly performed by Dr. Alice Harmon (University of Florida, Gainesville, FL) using a mixture of monoclonal antibodies against soybean CDPK or by us with polyclonal antibodies against *Arabidopsis* CDPK obtained from transformed *E. coli*, kindly provided by Dr. Jeffrey Harper (The Scripps Research Institute, San Diego, CA).

RESULTS AND DISCUSSION

Plasma Membrane H^+-ATPase Activity. The protein content and H^+-ATPase activity of plasma membrane (PM) from muskmelon hypodermal-mesocarp were highest in preharvest (mature) versus 1 day after abscission (harvested) or after 7 days of fruit storage (stored) (Table 1). The total decline in muskmelon fruit PM protein content in mature versus stored fruit was

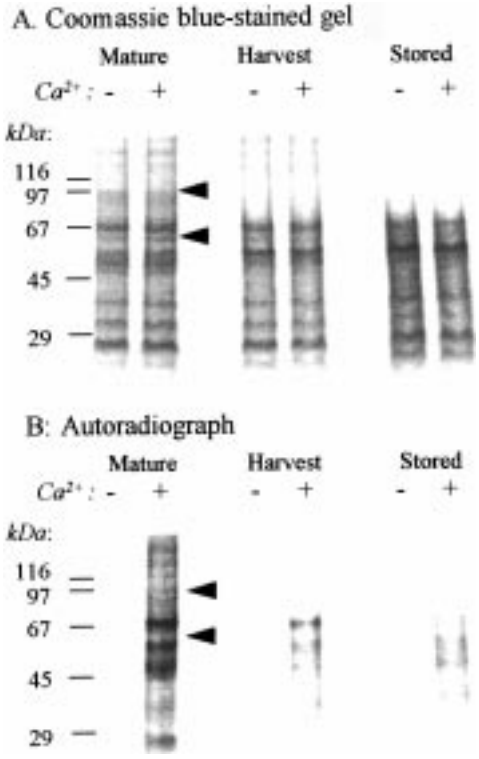

Figure 1. Phosphorylation of preharvest-mature (mature), harvested, and stored muskmelon hypodermal-mesocarp plasma membranes by endogenous kinases. Plasma membrane vesicles (15 (μg of protein)·well^{-1}) were phosphorylated for 5 min in the absence or presence of 20 μmol of free Ca^{2+}. A is a photograph of a Coomassie Blue-stained gel, and B is an autoradiograph. The position of the molecular weight markers (kDa) are indicated in the left margin. The upper arrow indicates the position of the 97 kDa protein, the region of all known H^+-ATPases, and the lower arrow indicates the position of the 63 kDa protein, the region of calcium-dependent protein kinase.

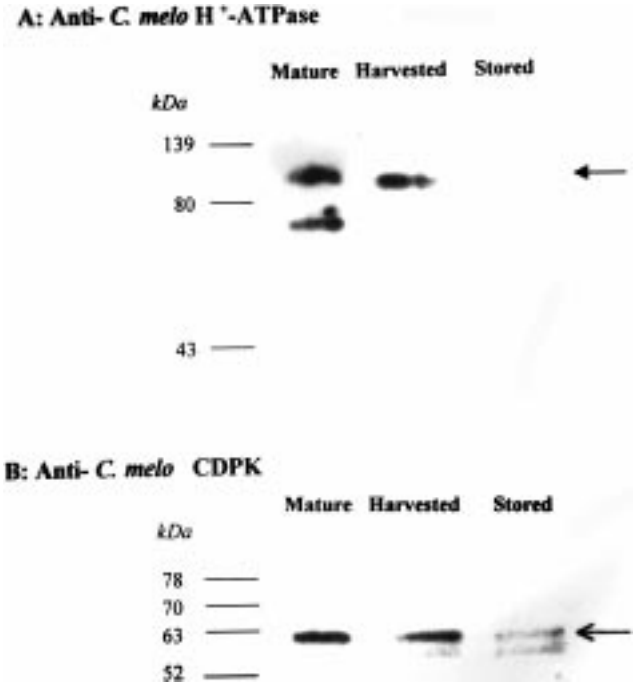

Figure 2. Proteins of muskmelon plasma membrane and their interaction with P-type H^+-ATPases and calcium-dependent protein kinase antibodies. Hypodermal-mesocarp plasma membrane proteins from preharvest-mature (mature), harvested, and stored melons were separated on SDS−PAGE, then blotted on nitrocellulose membranes, and probed with H^+-ATPases (A) and calcium-dependent protein kinase (B) antiserums, respectively. The arrow in (A) points to the 97 kDa band corresponding to P-type H^+-ATPases. The arrow in (B) points to the 63 kDa band corresponding to calcium-dependent protein kinase.

17%, whereas H^+-ATPase specific and total activity decreased by 71% and 76%, respectively. This decline in protein content and H^+-ATPase activity corroborated a previous report showing a decline in these physicochemical factors following muskmelon fruit maturation and postharvest senescence (Lester and Stein, 1993) and confirmed the implication that H^+-ATPase activity is a reliable measure of fruit PM senescence (Marangoni et al., 1996).

Polyacrylamide Gel Electrophoresis and Immunodetection. A Coomassie blue stained SDS gel of fruit PM proteins (Figure 1A) showed that free Ca^{2+} during the protein kinase reaction to determine kinase Ca^{2+} dependence (Figure 1B) did not affect either the protein profile or protein staining intensity of PM proteins, regardless of fruit maturity or degree of senescence. Following fruit abscission the protein profile in harvested and stored fruit PM (Figure 1A) declined in high (>67 kDa) molecular weight bands. Among these protein bands, is a faint band at 97 kDa, in mature fruit PM corresponding to the molecular weight of all previously reported H^+-ATPases (Schaller and Sussman, 1988). Immunodetection of this peptide with PM H^+-ATPase antibody recognized two polypeptides in mature, one in harvested, and none in stored fruit PM (Figure 2A). One band at 97 kDa was present in both mature and harvested fruit PM. The double band, which has been detected in other plant species (Baur et al., 1996) was visible only in mature melon fruit PM and may have resulted from isoforms of the enzyme with different SDS gel mobility. Another possibility is that specific proteolysis activated the enzyme and produced a double band on SDS gels by removal of the terminal segment in a subpopulation of the enzyme peptide (Baur et al., 1996). Our melon PM 97 kDa SDS gel (H^+-ATPase) band was not detected in stored fruit; this indicative loss of polypeptide content coincided with the loss in specific and total enzyme activity (Table 1).

Kinase Activity. The autoradiograph following protein kinase reaction, with and without Ca^{2+}, indicated that phosphorylation of muskmelon fruit PM proteins occurred only in the presence of Ca^{2+} (Figure 1B). This phosphorylation of PM proteins, which was more intense in mature attached fruit versus harvested or stored fruits, confirmed the presence of kinase. Immunodetection of this peptide with PM kinase antibody recognized a polypeptide band at 63 kDa that was visible in mature, harvested, and stored fruit, although the intensity decreased with fruit abscission and storage (Figure 2B). Phosphorylation of H^+-ATPase (97 kDa polypeptide) appeared detectable only in mature fruit PM.

Kinase activity in stored versus mature preharvest fruit decreased by 93% (Table 2). Incubating PM from stored fruit in the presence of 20 μmol of free Ca^{2+} showed a 12-fold increase in kinase activity. Thus, the observed decline in kinase activity in harvested and stored fruit may be due more to a decrease of a necessary substrate than to a loss of the kinase polypeptide. To test for this possibility, kinase assays were done with histone III−S, a protein known as a good substrate for PM kinase (Baizabal-Aguirre and Gonzalez

Table 2. Specific Protein Kinase Activity ((nmol of ^{32}P Incorporated)·min^{-1}·(mg of Protein)$^{-1}$) in the Presence or Absence of Histone III−S and/or Ca^{2+} of Var. Cruiser Muskmelon Hypodermal-Mesocarp Plasma Membrane from Mature (2 Days prior to Abscission), Postharvest (30 h at 24 °C), and Storage (7 Days at 21 °C plus 90 ± 5% RH)

fruit tissue maturity	control	Ca^{2+} (20 μmol of free Ca^{2+})	histone III−S (1 mg·mL^{-1})	Ca^{2+} plus histone III−S
mature	0.128 ± 0.060[a]	0.731 ± 0.377	0.710 ± 0.288	2.174 ± 0.091
postharvest	0.108 ± 0.013	0.132 ± 0.018	0.858 ± 0.022	1.279 ± 0.066
storage	0.003 ± 0.001	0.036 ± 0.058	0.966 ± 0.300	1.017 ± 0.031

[a] Means $n = 3 \pm$ sd.

Table 3. Specific Protein Kinase Activity ((nmol of ^{32}P Incorporated)·min^{-1}·(mg of Protein)$^{-1}$) of EGTA Washed Plasma Membranes in the Presence of Calcium (20 μmol of Free Ca^{2+}) and Histone III−S (1 mg·mL^{-1}) and/or Absence of Calmodulin (1 mg·mL^{-1}) of Var. Cruiser Muskmelon Hypodermal-Mesocarp Tissue from Mature (2 Days prior to Abscission), Postharvest (30 h at 24 °C), and Storage (7 Days at 21 °C plus 90 ± 5% RH)

fruit tissue maturity	Ca^{2+} + histone III−S	Ca^{2+} + histone III−S plus calmodulin
mature	1.577 ± 0.149[a]	2.124 ± 0.098
postharvest	1.181 ± 0.199	1.665 ± 0.332
storage	1.351 ± 0.427	1.331 ± 0.372

[a] Means $n = 3 \pm$ sd.

de la Vara, 1994). With this substrate, kinase activity increased 5.5-, 7.9-, and 322-fold for preharvest-mature, harvested, and stored fruit PM, respectively, indicating that a required kinase substrate continuously decreased with time after melon harvest. A suitable melon kinase substrate, H^+-ATPase, declined in harvested and stored fruit to undetectable levels, as indicated by a loss in the 97 kDa band (Figures 1A and 2A).

A stimulating effect of calmodulin on kinase activity was observed for all fruits by washing PM with EGTA to remove Ca^{2+} and Ca^{2+}-binding proteins, e.g. calmodulin, from the membrane (Collinge and Trewavas, 1989), and incubating in Ca^{2+} plus or minus calmodulin (Table 3). Comparison of non-EGTA washed PM (Table 2) with washed PM (Table 3) demonstrated that washing decreased kinase activity only in the PM from preharvest-mature and harvested fruits. This decrease was not observed when calmodulin was present in the assay. In stored fruits, a 30% increase in protein kinase activity was observed after EGTA washing. This increased kinase activity could indicate the presence of an unknown kinase inhibitor whose concentration increased with time after muskmelon fruit harvest or may be an anomaly due to the large sd. Calmodulin is an activator of PM-type ATPases and kinases (Leshem, 1992). In agreement with this, our results showed that preharvest mature melon fruit PM kinase activity is stimulated by calmodulin plus Ca^{2+}, although a loss in sensitivity occurs in postharvest fruit.

Muskmelon fruit PM has a high concentration of palmitic (C16:0), linoleic (C18:1), and linolenic (C18:2) acids at maturity, and a decline in their concentration occurs in postharvest fruit (Lester, 1990). Palmitic acid has been reported to be an inhibitor/activator of kinase activity (Minichiello et al., 1989). When muskmelon PM from mature, harvested, and stored fruits were washed with fatty acid−free serum albumin, which complexes with free fatty acids, and then incubated with palmitic acid, no effect on the kinase activity was observed (data not shown). In contrast with this result, C16:0, when incubated with Ca^{2+}, activates wheat (*Triticum aestivum* L.) and beet (*Beta vulgaris* L.) PM kinases (Polya et al., 1990).

Our study is the first attempt to show highly likely calcium-stimulated protein kinase phosphorylation of PM H^+-ATPase in muskmelon fruit. However, the loss in H^+-ATPase activity which occurred in postharvest fruit is most probably due to a decreased polypeptide content rather than decreased phosphorylation. Although kinase activity decreased in postharvest fruits, it did not appear to be due to a loss in its protein content, especially in harvested fruits, but rather to a loss of a necessary substrate, such as H^+-ATPase, plus a decrease in PM calcium content (Lester, 1996). Therefore, the heightened effect of Ca^{2+} on H^+-ATPase activity observed in harvested and stored fruits is most likely through maintenance of the structural integrity of the plasma membrane and not due to heightened kinase activity.

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