Changes in parameters of the plasmalemma ATPase during peach vegetative bud dormancy

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Abstract

Plant dormancy and dormancy breaking depend, at least partially, on close relationships between buds and tissues underlying bud (bud stands). In *Prunus persica*, the dormancy was related to high nutrient absorption in bud stands linked to high plasmalemma ATPase (EC 3.6.1.3) activity. Two plasmalemma fractions was isolated from peach vegetative buds and bud stands using aqueous phase partitionning and ultracentrifugation. Results of markers enzyme assays indicated that both plasmalemma enriched fractions obtained were highly purified. During the dormancy period plasma membrane ATPase amount and activity were higher in bud stands than in buds. Moreover, assays performed at different temperatures (4, 18, 30 °C) indicated modifications of kinetic parameters (K_m, V_m) in both tissues during dormancy release. In buds, from November to February, K_m declined at 4 °C and increased at 30 °C whereas no changes was measured at 18 °C and V_m increased at all temperature. In bud stands, no changes of K_m was measured at 4 °C and 18 °C whereas an increase occurred at 30 °C and V_m decreased at all temperature. According to the results, it can be postulated that dormancy release in peach-tree could be related to modifications of plasma membrane ATPase properties, in buds and bud stands, during winter time.

Additional key words: bud, H^+-ATPase activity, K_m, plasma membrane, *Prunus persica*, V_m.

Introduction

Plasmalemma ATPase plays a central role in plant physiology. The proton gradient generated by the enzyme is the driving force for active nutrient transport, and the pH changes resulting from proton pumping may be involved in growth control (Serrano 1989). Involvement of plasma membrane H^+-ATPase in plant dormancy was previously observed in Jerusalem artichoke tubers (Pétel and Gendraud 1986). The underlying parenchyma of the bud was characterized by a higher plasmalemma ATPase activity in dormant tissues compared to non-dormant parenchyma. This was interpreted as higher nutrient absorption potential in dormant parenchyma, leading to a situation of relative carency of bud, thus inhibiting its growth (Gendraud and Pétel 1990). In this way, plasmalemma ATPase activity can, at least partially, define "sink" and "source" regions. In relation to peach bud dormancy, modifications of active sucrose absorption were observed in bud and its underlying tissues, bud stand (also called cushion) and stem (Marquat *et al.* 1996). This phenomenon could also involve plasma membrane H^+-ATPase. In the studies presented, two plasmalemma fractions were prepared from buds and bud stands and evaluated for purity. ATPase activity, ATPase amount and kinetics of the protein were examined in relation to vegetative cycle.

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Abbreviations: BCIP - 5-bromo-4-chloro-5-indolyl phosphate; NBT - nitroblue tetrazolium; PAGE - polyacrylamide gel electrophoresis; PEG - polyethylene glycol; PMSF - phenylmethanesulfonyl fluoride; PVP - polyvinylpolypyrrolidone; TBS - tris buffer saline.

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Materials and methods

Plant material: Peach (*Prunus persica* cv. Dixired) buds and tissues underlying buds (bud stands) were harvested and peeled off just before use every month from November to March. All materials were washed twice with water and once with distilled water.

Isolation of plasma membrane: Aqueous two-phase partition was performed as follows. A thousand of peeled buds (about 2 g) or bud stands (about 3 g) was ground in 10 cm^3 of 50 mM Hepes buffer (pH 7.8) with 10 % PVP (m/v), 0.5 M sucrose, 5 mM ascorbic acid, 3.6 mM cysteine and protectants to avoid proteolysis (50 µg cm^{-3} chymostatin, 5 mM EDTA and 1 mM PMSF), filtered through four layers of cheesecloth and then centrifuged once for 10 min at 3 500 *g* and once at 20 000 *g* for 30 min to eliminate debris, mitochondria and plastids. The supernatant was centrifuged for 2 h at 100 000 *g*. The microsomal fraction was resuspended in 1 cm^3 of phosphate buffer (5 mM K_2HPO_4, pH 7.8 with KH_2PO_4) with 0.3 M sucrose, 3 mM KCl and 1 mM PMSF. One gram of the resuspended pellet was added to a 14 g phase mixture with the final composition of 6 % dextran *T-500* (m/v) (*Sigma*, St. Louis, USA), 6 % PEG 3350 (m/v) (*Sigma*), 0.3 M sucrose, 1 mM KCl and 5 mM phosphate buffer. After mixing, the system was centrifuged at 3 000 *g* for 5 min. The upper phase was separated, diluted with one volume of phosphate buffer and centrifuged for 3 h at 100 000 *g*. The final pellets, corresponding to the plasmalemma enriched fraction was taken up in a resuspension buffer (25 mM Hepes-NaOH, pH 7.5, 0.3 M sucrose, 50 mM KCl, 1 mM PMSF, 1 mM DTT) and stored at -20 °C until use. All steps were performed at ice temperature.

Preparation of antibodies: Polyclonal antibodies against H^+-ATPase from peach-tree were obtained by injecting two white female rabbits with two doses of the purified enzyme obtained from peach-tree buds. The serum collected was precipitated with 40 % ammonium sulfate and kept at -25 °C before used.

Electrophoresis and Western blot: SDS-PAGE electrophoresis was performed using normal (1 × 140 × 250 mm) discontinuous polyacrylamide gels (Laemmli 1970). Proteins were separated on a 12 % resolving gel at constant voltage (150 V) for about 15 h. Fifteen µg of proteins were applied per sample well and protein staining was done with silver nitrate. Immunodetection in Western blot was made with TBS as the basic medium. Teleostan gelatin 3 % (*Sigma*) and Tween 20 (0.75 cm^3 dm^{-3}) were used in the blocking of nitrocellulose. Polyclonal antibodies against peach ATPase was diluted to 1/1 000 and the second antibody, conjugated to alkaline phosphatase, was diluted to 1/3 000 (monoclonal anti-rabbit IgG alkaline phosphatase conjugate, immunoglobulin fraction of mouse ascites fluid, clone RG96 (*Sigma*). The phosphatase alkaline substrates were NBT and BCIP. Quantitative analysis of the western blot was made using a *Shimadzu* dual wavelength scanner *CS 9000* at 535 nm.

Enzyme assays: ATPase assays were performed by bioluminescence as previously described (Pétel and Gendraud 1986) using an aliquot of membrane preparation containing 15 µg protein. Characterization assays and assays for specific activity along dormancy were conducted at 18 °C while kinetic determinations during dormancy were performed at 4, 18, and 30 °C. Kinetic constants (K_m, V_m) were determined from Lineweaver and Burke representation of ATPase activity over a substrate concentration range from 0.125 to 4 mM ATP. Triplicate assays were conducted for each concentration of ATP, and the representations constructed from activity data had correlation coefficients ranging from 0.97 to 0.99.

Cytochrome *c* oxidase activity measurement was adapted from Applemans *et al.* (1955), modified by Le Floc'h and Lafleuriel (1983). The reaction medium contained 30 mM phosphate buffer (pH 7.4), 1 mM EDTA and 50 µM reduced cytochrome *c*. The reaction was initiated by addition of plasma membrane fraction and the decrease of absorbance at 550 nm was measured.

Chlorophyll and protein content: Chlorophyll content was determined by the method of Mac Kinney (1941). An aliquot of membrane preparation (0.02 cm^3) containing approximately 15 µg protein was mixed with 0.18 cm^3 of distilled water and 0.8 cm^3 of acetone and then centrifuged at 10 000 *g* for 15 min. Absorbance of the upper phase was measured at 652 nm and total chlorophyll concentration (mg cm^{-3}) was estimated (total chlorophyll = 0.029 A_{652}).

Protein content was determined by the Bradford method (1976), using bovine serum albumin as a standard.

Results

Purity of the plasma membrane fractions: To characterize the vesicles isolated by aqueous two-phase partitioning from buds and bud stands, the effects of various ATPase effectors and others markers were studied (Table 1). It was found that ATPase activity, in both buds and bud stands fractions, was not stimulated by KCl but strongly inhibited by vanadate to 88 % and 87 %, respectively. No significant inhibition by nitrate was recorded in both fractions. Finally, no cytochrome *c* oxidase activity and chlorophyll content were found, indicating that both fractions were free from mitochondria and thylakoid contamination. No variation of purity was measured between fractions obtained from plant material harvested at different dates (data not shown).

Table 1. Plasmalemma enriched fractions characterization. Plasmalemma fractions were obtained from plant material harvested at the end of February. Means of 5 measurements ± SE. No cytochrome *c* oxidase activity and chlorophyll content was detected.

ATPase activity	Bud stands [nkat mg^{-1}]	[%]	Vegetative buds [nkat mg^{-1}]	[%]
Control	1.05 ± 0.05	100	1.62 ± 0.07	100
+KCl	1.09 ± 0.06	104	1.63 ± 0.03	101
$+KNO_3$	1.06 ± 0.06	101	1.61 ± 0.05	99
$+VO_4$	0.14 ± 0.02	13	0.19 ± 0.05	12
Amount of protein [µg]	109 ± 10		116 ± 13	

Evolution of the ATPase activity during vegetative cycle: Specific ATPase activity was measured from plasma membrane fractions regularly obtained from buds and bud stands during vegetative cycle (Fig. 1). In bud stands, specific ATPase activity decreased from 1.5 to 1 nKat mg^{-1}(protein) from November through mid-December and then increased until March from 1 to 1.4 nKat mg^{-1}(protein). In buds, ATPase activity increased linearly from 0.8 to 1.6 nkat mg^{-1}(protein) from November through March and was less than in bud stands until mid-December.

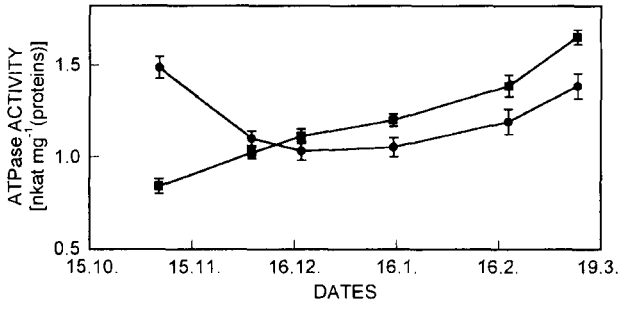

Fig. 1. Plasma membrane H^+-ATPase activity measured in buds (*squares*) and bud stands (*circles*), during peach vegetative buds dormancy. Means of 5 measurements ± SE.

Quantitative analysis of plasmalemma ATPase: Quantity of ATPase was immunologically estimated using polyclonal antibodies obtained from peach buds. Antibodies react with one polypeptide with molecular mass of 95 kDa on the pattern of plasma membrane enriched fraction obtained from buds or bud stands (Fig. 2). No other polypeptides were labelled when using extracts obtained at other dates (data not shown). Quantitative analysis was made using the same fractions obtained for specific ATPase activity analysis (Fig. 3). In buds, from November through mid-December, ATPase quantity increased from 0.18 to 0.55 A_{535}, while it decreased in bud stands from 0.63 to 0.42 A_{535}. From January to March, ATPase quantity remain constant in both tissues, with a higher level in buds.

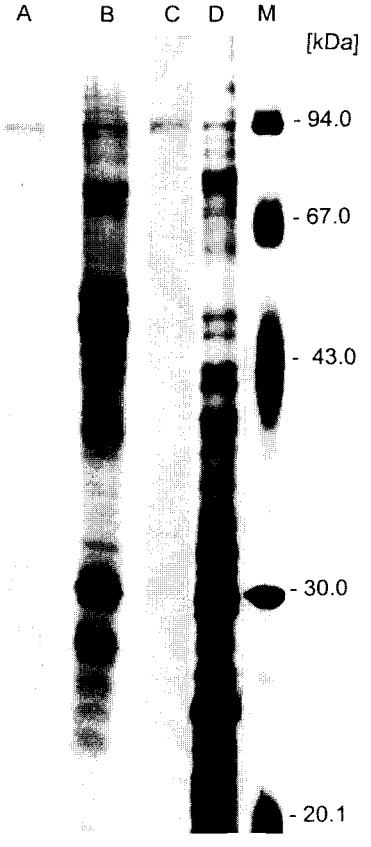

Fig. 2. Specificity of the polyclonal antibody against the peach H^+-ATPase. SDS-PAGE electrophoresis of plasma membrane enriched fraction from buds (*B*) or bud stands (*D*) and western-blot of plasma membrane enriched fraction from buds (*A*) or bud stands (*C*) using antibodies against H^+-ATPase from peach. Plasmalemma fractions were obtained from plant material harvested at the end of February. *M* - molecular mass standards.

Plasmalemma ATPase kinetics determination: Kinetics (V_m, K_m) of the plasma membrane ATPase were determined at different temperatures using three plasma membrane fractions obtained from buds and bud stands at different dates during vegetative cycle. In buds (Table 2), assays conducted at 4 °C indicated that K_m declined significantly from 0.87 to 0.54 during vegetative cycle. No modifications of K_m was measured when assay was performed at 18 °C while assays conducted at 30 °C showed an increase of K_m from 1.02 to 1.22. Assays conducted at 4, 18 or 30 °C indicated a linear increase of V_m, in buds, during vegetative cycle. In bud stand (Table 3), assays conducted at 4 °C indicated a little decrease of K_m from 0.97 to 0.90 while when assays was performed at 18 °C K_m increased from 0.99 to 1.11 from November to mid-December and then decreased from 1.11 to 1.01 until February. At 30 °C, K_m regularly increased during vegetative cycle. Assays conducted at 4, 18 or 30 °C in bud stands indicated a linear decrease of V_m during vegetative cycle.

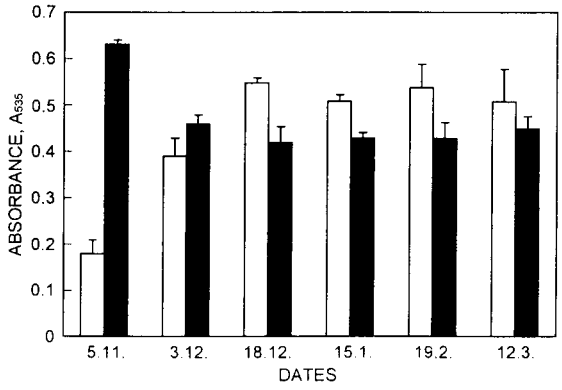

Fig. 3. Immunological quantification of plasma membrane H^+-ATPase in buds (*open columns*) and bud stands (*closed columns*) during vegetative buds dormancy. *Bars* represent SE of 3 measurements.

Table 2. Plasmalemma ATPase kinetics of buds and bud stands determined from assays performed at 4, 18, or 30 °C at different dates during dormancy. Means ± SE of three experiments.

		K_m [mM]			V_m [nkat mg^{-1}(protein)]		
		4 °C	18 °C	30 °C	4 °C	18 °C	30 °C
Buds	5 November	0.87 ± 0.03	0.95 ± 0.02	1.02 ± 0.02	0.36 ± 0.03	2.03 ± 0.03	5.26 ± 0.10
	18 December	0.70 ± 0.02	0.96 ± 0.01	1.15 ± 0.02	0.47 ± 0.02	2.24 ± 0.06	6.44 ± 0.14
	19 February	0.54 ± 0.01	0.95 ± 0.02	1.22 ± 0.05	0.55 ± 0.01	2.80 ± 0.08	7.69 ± 0.11
Bud	5 November	0.97 ± 0.04	0.99 ± 0.02	1.04 ± 0.04	0.53 ± 0.03	2.98 ± 0.07	7.60 ± 0.17
stands	18 December	0.95 ± 0.02	1.11 ± 0.02	1.15 ± 0.03	0.42 ± 0.01	2.39 ± 0.04	5.84 ± 0.12
	19 February	0.90 ± 0.03	1.01 ± 0.03	1.33 ± 0.06	0.31 ± 0.02	1.90 ± 0.05	4.71 ± 0.15

Discussion

Dormancy could be related to high nutrient absorption potential in buds underlying tissues: this was observed in Jerusalem artichoke tubers (Gendraud and Lafleuriel 1983), as well as in peach (Marquat *et al.* 1996). In Jerusalem artichoke, it was linked to high cytoplasmic pH of underlying tissue, related to a higher plasmalemma ATPase activity (Pétel *et al.* 1992). In peach, the plasma membrane ATPase activity and kinetics of vegetative buds and bud stand during the rest period and dormancy release determine. For this purpose, the acquisition of a pure plasma membrane fraction from vegetative buds and bud stands was needed. Vanadate-sensitive ATPase activity of the fractions represented from 87 to 88 % of phosphatase activities. This can be compared to results obtained on other plants (Gallet *et al.* 1989, Mito *et al.* 1988). Nitrate-sensitive ATPase activity was not significant in these fractions, indicating no contamination by tonoplast. In a similar way, no cytochrome *c* oxidase activities and chlorophyll amounts were detected. This indicated that the used fractions were free from mitochondria and thylakoids.

During dormancy (until mid-December), plasma membrane ATPase activity and quantity were higher in bud stands than in buds. This situation reveals higher metabolite absorption potential in bud stands, compared to buds. The bud stands could so be considered as a barrier, inducing a situation of relative carency of buds, thus inhibiting their growth. An inversion of this situation was observed after mid-December at the time the "cutting test" method indicated that dormancy was released (Balandier *et al.* 1993). At this time, ATPase activity and quantity became higher in buds than in bud stands, indicating that nutrients could then supply buds. This interpretation was supported by the measurements of sucrose absorption made in the same tissues (Marquat *et al.* 1996). In November, the active sucrose absorption in

bud stands, involving ATPase activity as an energy source, is twice that in buds and a reversion of sucrose absorption between these tissues was also noted when dormancy was released. Changes in the kinetic constants of the plasma membrane ATPase also occur during vegetative cycle. Modifications of V_m in both tissues could be related to respective modifications of ATPase quantity but K_m changes which differ depending on the assay temperature and the tissue studied indicated alterations in ATPase properties during vegetative cycle. Dormancy release appears to result in more efficient ATP catalysis at low temperature than at high temperature in buds while no significantly changes occur in bud stands. The decrease in K_m values at 4 °C may result in enhanced capacity for low-temperature solute transport inducing the reacquisition of metabolites absorption capabilities by buds. These changes in kinetic of plasma ATPase between buds and bud stand could be related with changes in membrane fluidity measured between the same tissues during dormancy in peach (Portrat *et al.* 1995) but alterations in the ATPase protein itself could be envisaged. Dormancy release of vegetative buds appears to be partially caused by a modification of plasma membrane ATPase quantity correlated with modifications of kinetic parameters of the protein in both buds and bud stands, leading to a reorientation of metabolites fluxes. The consequence will be the breaking of short distance correlative inhibitions, as indicated by the "cutting test" method. According to this hypothesis, it can be postulated that dormancy release is linked to the modification of plasma membrane H^+-ATPase properties, in both buds and bud stands, during winter time.

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