

Selective Accumulation of the Fluorescent pH Indicator, BCECF, in Vacuoles of Maize Root-Hair Cells

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Summary

The vacuolar-type H^+ -ATPase localized on the tonoplast membrane is believed to regulate acidity within the vacuolar lumen. However, the ability to monitor vacuolar pH *in situ* can be difficult because of the use of destructive assays based on cell sap pH or accumulation of weak bases, or elicitation of wound responses after impaling cells with a pH-sensing microelectrode. The goal of our present study was to identify a fluorescent pH probe that is localized in vacuoles of (*Zea mays* L.) root cells. When excised corn root segments were exposed to the ester derivative of 2',7'-bis-2-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) for 30 to 60 min, the root-hair cells preferentially became fluorescent. The distribution of the fluorescence within cells followed closest to that of the vacuole when fluorescent and phase contrast images were compared. The ratio of the fluorescence intensities at 535 nm using excitation wavelengths of 440 and 490 nm varied directly with pH between 4.5 and 7.5. The ratio of fluorescent intensities of BCECF-labeled root-hair cells indicated that the majority of the dye was localized in a compartment with an average pH of 5.8. The above data indicated that the BCECF was localized in the vacuoles of root-hair cells from maize seedlings, and the changes in the fluorescence ratio of BCECF may provide a means of monitoring changes of vacuolar pH *in situ*.

Key words: Lipophilic precursor, microscopy, ratio imaging, tonoplast, *Zea mays* L.

Abbreviations: BCECF = 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BCECF-AM = acetoxymethyl ester of BCECF; fluorescence ratio (440/490) = the ratio of fluorescent intensities at 535 nm with an excitation wavelength of 440 nm to that obtained with an excitation wavelength of 490 nm.

Introduction

The biochemistry and regulation of the P-type and V-type proton pumps from corn roots has been an area of research of our laboratory. One objective of our research has been the study of the actions of these two proton pumps *in situ*. One means by which the net transport of protons in and out of the cytoplasm can be followed *in situ* is the use of *in vivo* ^{31}P NMR (Pfeffer et al. 1987, Roberts et al. 1981) because the chemical shift of inorganic phosphate is pH dependent (Roberts et al. 1980). Typically, ^{31}P -NMR spectra of plant cells contain two peaks corresponding to two pools of inorganic phosphate differing in pH (Pfeffer et al. 1987, Roberts et al. 1981). The acidic pool has been assigned to the vacuole

whereas the slightly alkaline pool has been assigned to the cytosol. The pH of the cytosol can be determined quite precisely because of the relatively large chemical shift in the inorganic phosphate between pH 6 and 8 (Pfeffer et al. 1987). The pH of the vacuole can not be evaluated as precisely by *in vivo* ^{31}P -NMR because of a smaller change in the chemical shift of inorganic phosphate between pH 4 and 6 (Pfeffer et al. 1987).

Other techniques have been used to estimate vacuolar pH, but these techniques can have serious limitations. Some researchers have estimated vacuolar pH by determining the pH of tissue sap (Kurkdjian et al. 1985). This technique will yield an estimate of vacuolar pH only if the buffering capacity of the non-vacuolar cell compartments is relatively low

as compared with the constituents within the vacuole. Other researchers estimated vacuolar pH by following the accumulation of radioactive lipophilic weak bases (Kurkdjian and Guern 1981, Kurkdjian et al. 1985, Marre et al. 1986). In theory, these compounds will be trapped in the most acidic cell compartment, presumably the vacuole, as dictated by the Henderson-Hasselbach equation. However, the vacuole may not be the site of accumulation if the base is metabolized or binds significantly to other cell components (Kurkdjian and Guern 1981, Kurkdjian et al. 1985). The above techniques including *in vivo* ^{31}P NMR have limited time resolution, and the results are the average pH values among the different cell types in the tissues. Changes in cytoplasmic and vacuolar pH can be monitored directly on individual cells with almost real time resolution after impaling with a pH microelectrode (Kurkdjian and Barbier-Brygoo 1983). With certain cells, however, impaling with the electrode elicits a wound response, inducing significant changes in cellular metabolism and possibly pH (Heiple and Taylor 1980, Talbot et al. 1988).

The use of fluorescence microscopy after the incorporation of pH sensitive dyes within the vacuolar lumen appears to be one technique that would allow both spatial and time resolution, in which injury to the tissue may not be necessary. The use of fluorescent probes to study the biochemistry of plants by fluorescence microscopy has been problematic in the past because of limitations in loading plant cells with dyes as lipophilic precursors. Entrapment of the lipophilic precursor as an optically active compound is dependent on the presence of esterases to remove the blocking groups. Selective entrapment of lipophilic precursor in vacuoles may be possible since subcellular compartmentation studies have indicated that the majority of esterase activity is localized in the vacuole of plant cells (Boller and Kende 1979). The results described in this report indicated that the lipophilic precursor of BCECF was preferentially accumulated in vacuoles of root-hair cells of maize seedlings. These results are novel because of the specific incorporation of a pH sensing dye within a specific subcellular compartment of a particular cell type within a plant organ.

Materials and Methods

Growth Conditions

Two- to three-day old seedlings of maize cultivars (*Zea mays* L. cv. FRB 73 from Illinois Foundation Seed Co and W7551 from Custom Farm Seed Co)¹ were germinated on paper moistened with 0.1 mM CaCl_2 (Brauer et al. 1989).

Loading of Roots with BCECF

The apical 3 to 4 cm of roots were excised from the seedlings and placed in 20 mL of perfusion solution containing 0.2 mM CaSO_4 , 50 mM glucose and 10 mM Mes-Hepes (pH 6.0). The solution was continuously bubbled with air. The BCECF~AM was dissolved in DMSO and the perfusion solution was adjusted to be $3\mu\text{M}$

¹ Mention of a brand or trademark does not constitute an endorsement of a product over similar items by United States Department of Agriculture.

BCECF~AM. The DMSO content of the perfusion buffer never exceeded 2% of the total solution volume. After 30 min of incubation at room temperature, roots were rinsed three times for 2 min each in 0.1 mM CaCl_2 . Roots were then held until used (< 2 h) in aerated pH 6.0 perfusion buffer.

Phase Contrast and Fluorescence Microscopy

Roots were visualized with a Nikon Diaphot inverted microscope equipped with long working distance phase contrast lens. Sample chambers were fashioned from plastic petri dishes by etching out a slot on the bottom and then cementing a sufficiently large 1.5 mm thickness cover slip to close the opening. Roots were then positioned over the cover slip and held in place with the aid of a small amount of modeling clay. To maintain the viability of the excised roots, 20 mL of perfusion buffer were added to the petri dish and aerated throughout the experiment.

Fluorescence was detected using a CCD camera (Photometrics, Ltd.) cooled to -45°C and a shutter speed of 0.5 s. The visualization of total fluorescence from BCECF was determined by using excitation and emission filters with band passes of 473 to 496 and 515 to 545 nm, respectively. When BCECF was used as a pH-sensing probe, the above emission filter was used. However, two sets of images were obtained within seconds of each other using excitation filters of 440 ± 10 and 490 ± 10 nm. The sample was not exposed to the excitation light during the time between image acquisitions.

Both phase contrast and fluorescent images were captured, stored, and analyzed using IPLab software (Signal Analysis Corp.). This software utilizes 512×387 pixel images and a gray scale of 4096 shades. To use BCECF as a pH probe, a fluorescence ratio at the two excitation wavelengths was calculated as follows. The background level of fluorescence was subtracted from the images. Background fluorescence was determined by three methods. Images of labeled roots with no actinic light and unlabeled roots with actinic light were recorded. The intensity of fluorescence using the perfusion buffer as the image was also determined. The estimates of the background fluorescence intensity by these three methods were similar (approximately value of 50), and the differences in intensity between the 440 and 490 nm filters were minimal (data not shown). After the background fluorescence was removed from the images, the average intensity of fluorescence within a cell with an excitation wavelength of either 440 or 490 nm was determined using the IPLab software. The intensities values varied between 200 and 500 after background subtraction. Then, the intensities were divided to obtain the fluorescence ratio of (440/490).

Results

Preferential Localization of BCECF to Root Hair Vacuoles

When root segments incubated in the presence of BCECF~AM and washed free of the loading solution were viewed at low magnification, it was apparent that the root hair cells contained the vast majority of the BCECF (Fig. 1). When thin cross sections of BCECF-labeled roots were prepared and visualized by fluorescence microscopy, virtually all the fluorescence was in the epidermal layer and absent from the cortical cells below (data not shown).

Definite evidence for the localization of the BCECF to the vacuole was obtained by comparing side-view images of root hair cells obtained by phase contrast and fluorescence microscopy (Fig. 2). Areas of the cells that are cytoplasmically dense can be distinguished from areas that are primarily vac-

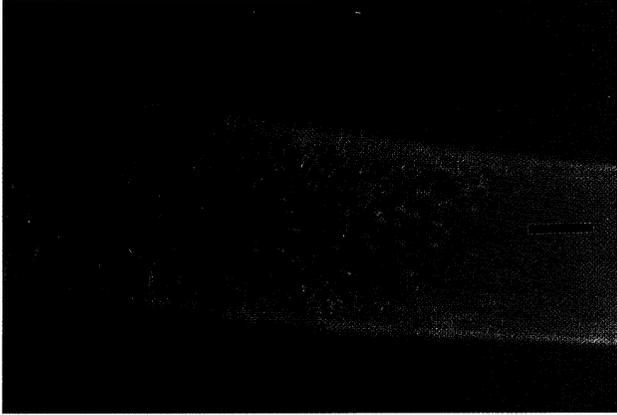


Fig. 1: Accumulation of BCECF in Root Hair Cells. Corn root segments were incubated in the presence of $4 \mu\text{g}/\text{mL}$ of BCECF ~ AM for 30 min at room temperature and then washed free of the loading solution. Labeled roots were visualized with fluorescence microscopy using excitation and emission wavelengths of 490 ± 10 and 535 ± 15 nm, respectively, with $4\times$ phase contrast objective. Total magnification of photographed image is $80\times$. Bar in the figure represents $200 \mu\text{m}$. Photographs were made using Ektachrome film (ASA 1600) and an exposure time determined by a Nikon UFX-DX camera controller.

uole by differences in refractive index as detected by phase contrast microscopy. With phase contrast (panel A), there were two areas on the right side of the root-hair projection that contained a relatively high density of cytoplasm. The cytoplasmic region toward the apex of the projection was the denser of the two. It should be noted that the rest of the

cellular volume is predominantly vacuole, but not exclusively. When monitoring regions of lower refractive index in cells for a few minutes, the movement of small organelles or vesicles was apparent, indicating the presence of cytoplasmic strands. The fluorescent image of the same root hair projection in panel B indicated that the cytoplasmically dense areas contain little if any fluorescence from BCECF. The fluorescence in the cell area containing mostly vacuole was of a rather uniform level with a few areas of more intense fluorescence. These areas of more intense fluorescence were associated with small vesicles apparently circulating within the cytoplasmic strands. The data compiled from studies of this nature indicated that areas of the cell enriched in cytoplasm were nearly devoid of BCECF while the majority of the dye was in the vacuole.

Calibration of BCECF as a pH probe

The fluorescence of BCECF at 535 nm is more sensitive to changes in pH when excited with light at 490 nm than when excited at 440 nm (Haugland 1992). An *in situ* calibration curve was constructed to relate the fluorescence ratio to pH in the presence of 50 mM of K^+ and 0.27 mM nigericin to equilibrate intracellular pH with pH gradients across that of the external solution (Tsein 1989). The length of incubation necessary for this equilibration was determined experimentally to be less than 10 min (data not shown). It was found that fluorescence ratio for BCECF in root hair cells varied with changes in pH between 4.5 and 7.5 (Fig. 3). The fluorescence ratio (440/490) of the root-hair cells examined thus far in this laboratory averaged 2.2 ± 0.05 ($n > 100$), corresponding to a pH of 5.8 ± 0.1 . Previously the average pH of

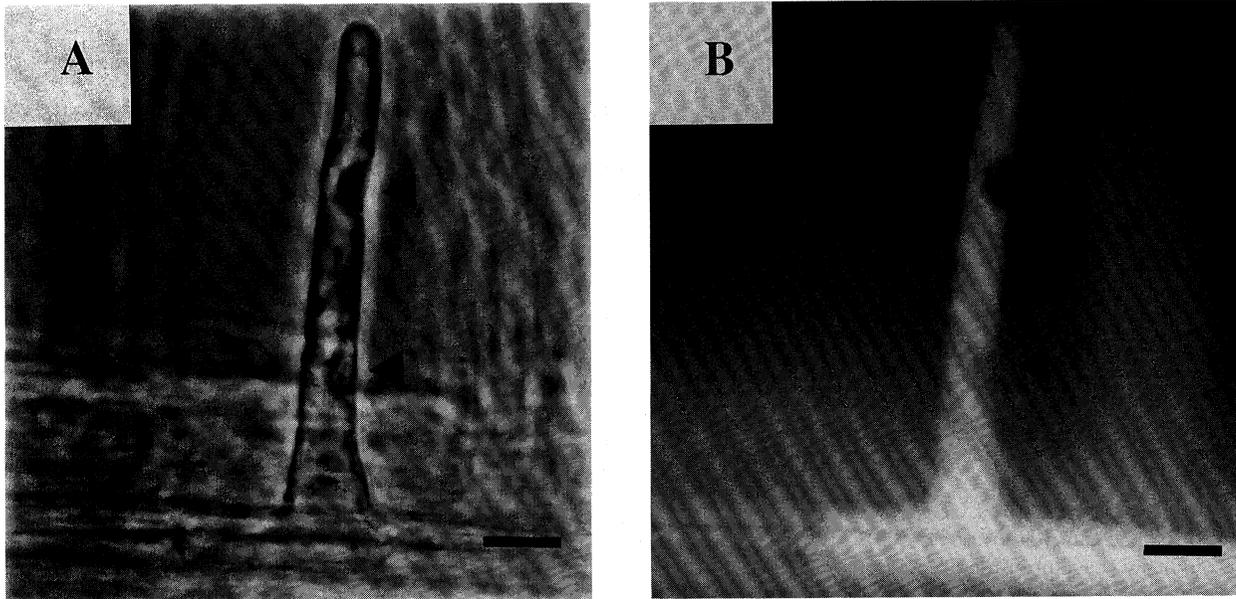


Fig. 2: Localization of BCECF to the vacuole of root hair cells. Corn root segments were labeled with BCECF as described in the Materials and Methods. Root hair cells were then examined in side views by phase contrast (panel A) and fluorescence (panel B) microscopy. Fluorescence microscopy was conducted with excitation and emission wavelengths of 490 and 535 nm, respectively. Bar in the figure represent $10 \mu\text{m}$. Total magnification of photographed image is $1400\times$.

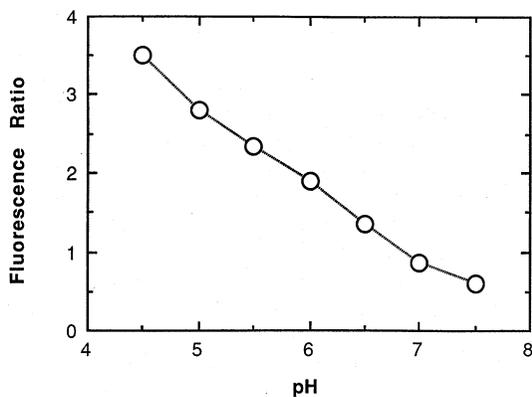


Fig. 3: Development of *in situ* calibration curve for BCECF. The fluorescence ratio of BCECF labeled root hair cells was determined 10 min after the addition of 50 mM KCl and 200 μ g nigericin/mL at varying external pH values (●).

this compartment in root tip tissue has been estimated to be between 5.5 and 6.0 by 31 P NMR spectroscopy (Pfeffer et al. 1987).

Discussion

Preferential Labeling of Root Hair Vacuoles by BCECF

The localization of the BCECF to the vacuoles in corn-root cells was not expected. BCECF~AM was developed to deliver BCECF to the cytoplasm of mammalian cells (Haugland 1992). In fact, BCECF~AM has been used to deliver the dye to the cytoplasm of plant cells, like suspension cultured *Catharanthus roseus* (Sakano et al. 1992), leaf cells of *Amaranthus caudatus* L. (Yin et al. 1993) and marine alga *Emiliania huxleyi* (Dixon et al. 1989). The reason for this apparent difference between maize and other tissues is not known, but could originate from differences in the subcellular distribution of esterases.

Another unexpected result in this research was the selective labeling of root hair cells. The reason for a particular cell type accumulating the dye also is not known. However, it would be sensible to postulate that selective absorption or metabolism of the precursor by the vacuoles in root-hair cells was responsible. Selective absorption of the dye precursor by root-hair cells could be related to the increased surface area of these cells relative to other epidermal cells. Differences in epidermal cells to accumulate BCECF~AM probably did not reflect differences in cell viability because all cells were able to exclude carboxyfluorescein and BCECF under loading conditions (data not shown). A pattern of accumulation similar to BCECF~AM was observed with fluorescein diacetate (data not shown). Preliminary experiments indicate that lipophilic precursors of carboxy-SNARF and carboxy-SNAFL are accumulated in the cytosol of all epidermal cells (data not shown). Similar result describing differences in the compartmentation of BCECF~AM and SNARF-1-AM have been reported recently with *Neurospora crassa* (Moussatos and Slayman 1993). Therefore certain

structures of these dyes must determine their fate in the tissue.

The loading of BCECF into a relatively small number of the epidermal cells had a decided advantage over uniform uptake of the dye. The selective accumulation of BCECF into the root hair cells resulted in images in which one could treat the fluorescence as originating from a discrete site. Therefore, the influence of out of focus fluorescent light was minimal, and corrections for such phenomenon were not necessary (Tsein 1989). Since the fluorescence ratio of BCECF varied with pH between 4.5 and 6.0, it appears to be useful for monitoring changes in vacuolar pH *in situ*. In addition, selective accumulation of the BCECF~AM in the vacuole enabled better estimates of the pH of this compartment than if the dye was distributed between the cytoplasm and vacuole. Two dimensional images represent light gathered from a three dimensional portion of the specimen of some definite, but hard to define, thickness. If the dye had accumulated in both compartments, one would need to estimate the portion of cytoplasm and vacuole in each region of a cell in order to interpret changes in fluorescence ratio with changes in pH. A better solution to the problem of simultaneously monitoring changes in cytoplasmic and vacuolar pH would be the use of 2 dyes, optically unique, each localized exclusively in one compartment. Such an approach may be feasible based on our initial results with BCECF~AM, and derivatives of SNARF and SNAFL.

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References

- BOLLER, T. and H. KENDE: Hydrolytic enzymes in the central vacuole plant cells. *Plant Physiol.* 63, 1123–1132 (1979).
- BRAUER, D. and S. TU: Kinetic analysis of proton transport by the vanadate-sensitive ATPase from maize root microsomes. *Plant Physiol.* 89, 464–471 (1989).
- DIXON, G. K., C. BRAUNTEE, and M. J. MERNETT: Measurement of internal pH in the coccolithophore *Emiliana huxleyi* using 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester and digital imaging microscopy. *Planta* 178, 443–449 (1989).
- HAUGLAND, R. P.: Handbook of fluorescent probes and research chemicals. Molecular Probes, Inc., Eugene, OR (1992).
- HEIPLE, J. M. and D. L. TAYLOR: Intracellular pH in single motile cells. *J. Cell Biol.* 86, 885–890 (1980).
- KURKDJIAN, A. and H. BARBIER-BRYGOO: A hydrogen ion-selective liquid-membrane microelectrode for measurement of the vacuolar pH of plant cells in suspension culture. *Anal. Biochem.* 132, 96–104 (1983).
- KURKDJIAN, A. and J. GUERN: Vacuolar pH measurement in higher cells. I. Evaluation of the methylamine method. *Plant Physiol.* 67, 953–957 (1981).
- — Intracellular pH: Measurement and importance in cell activity. *Annu. Rev. Plant Physiol.* 40, 271–303 (1989).

- KURKDJIAN, A., H. QUIQUAMPOX, H. BARBIER-BRYGOO, M. PEAN, P. MANIGAULT, and J. GUERN: Critical examination of methods for estimating the vacuolar pH of plant cells. In: MARIN, B. P. (ed.): *Biochemistry and Function of Vacuolar ATPase in Fungi and Plant cells*. Springer-Verlag, Berlin, pp. 98–113 (1985).
- MARRE, M. T., G. ROMANI, M. BELLANDO, and E. MARRE: Stimulation of weak acid uptake and increase in cell sap as evidence for fusicoccin- and K^+ -induced cytosol alkalinization. *Plant Physiol.* 82, 316–323 (1986).
- MOUSSATOS, V. V. and C. W. SLAYMAN: Endomembranal sequestration of fluorescent ion-indicator dyes in *Neurospora crassa*. *Plant Physiol.* 102, 109 (Abstract # 615) (1993).
- PFEFFER, P. E., S. TU, W. C. GERASIMOWICZ, and T. R. BOSWELL: Effects of aluminium on the release and/or immobilization of soluble phosphate in corn root tissue: A ^{31}P nuclear magnetic resonance study. *Planta* 172, 200–208 (1987).
- ROBERTS, J. K. M., P. M. RAY, N. WADE-JARDETZKY, and O. JARDETZKY: Estimation of cytoplasmic and vacuolar pH in higher plant cells by ^{31}P NMR. *Nature* 283, 870–872 (1980).
- — — — Extent of intracellular pH changes during H^+ extrusion by maize root-tip cells. *Planta* 152, 74–78 (1981).
- SAKANO, K., Y. YAZAKI, and T. MIMURA: Cytoplasmic acidification induced by inorganic phosphate uptake in suspension cultured *Catharanthus roseus* cells. Measurement with fluorescent pH indicator and ^{31}P -nuclear magnetic resonance. *Plant Physiol.* 99, 672–680 (1992).
- TSEIN, R. Y.: Fluorescent indicators of ion concentrations. In: TAYLOR, D. L. and Y. WANG (eds.): *Methods in Cell Biology*, Vol. 30. pp. 127–156 (1989).
- TALBOTT, L. D., P. M. RAY, and J. K. M. ROBERTS: Effect of indoleacetic acid and fusicoccin-stimulated proton extrusion on internal pH of pea internode. *Plant Physiol.* 87, 211–216 (1988).
- YIN, Z.-H., U. HEHER, and A. S. RAGHAVENDRA: Light-induced pH changes in leaves of C_4 plants. Comparison of cytosolic alkalination and vacuolar acidification with that of C_3 plants. *Planta* 189, 267–277 (1993).

Characterization of Thirteen Date Palm (*Phoenix dactylifera* L.) Cultivars by Enzyme Electrophoresis using the PhastSystem

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Summary

The analysis of date palm (*Phoenix dactylifera* L.) enzymatic polymorphism enabled us to establish some reference «identity cards» for the cultivars studied. Thirteen cultivars and ten enzymatic systems were investigated. Isozyme separation was performed with the PhastSystem (Pharmacia, Uppsala), an automated electrophoresis unit, allowing the standardization of migration parameters. Various zymograms have been obtained for five of the ten enzymes studied, showing differences in isozyme number, band intensities, and relative mobility values. We have found six polymorphic loci, expressing thirteen different alleles. These variations are only caused by genetic differences among the cultivars, enabling us to assemble some of them and accurately distinguish nine (69%) of the thirteen cultivars studied.

Key words: *Phoenix dactylifera* L., date palm cultivars, leaf isozymes, varietal identification, enzyme polymorphism, PhastSystem.

Abbreviations: BSA = Albumin bovine; EDTA = Ethylenediaminetetraacetic acid; PVP = polyvinylpyrrolidone MW = 40,000; Tris = Tris(Hydroxymethyl) Aminomethane.

Introduction

Date palm (*Phoenix dactylifera* L.) cultivar identification has been traditionally based on a combination of vegetative and fruiting characteristics. Hence, the different cultivars are sometimes difficult to distinguish from each other. The lack of a practical key for cultivar identification and the long life cycle of date palm promoted the need to establish appropriate and reliable methods of identification. Moreover, it should be interesting to characterize date palm cultivars at an earlier stage of their development. During the past years, isozyme analysis appeared to be a possible alter-

native or complementary method for cultivar identification. It has been successfully applied to such diverse species as various fruit trees (Weeden and Lamb, 1985; Menendez and Daley, 1986; Bournival and Korban, 1987; Hauagge et al., 1987), grape (Benin et al., 1988), potatoes (Oliver and Martinez-Zapater, 1985; Contreras and Mansilla, 1989), cereals (Cardy and Kannenberg, 1982; Lallemand and Briand, 1990), forest trees (Bergmann, 1987; Rajora, 1989), tropical crops (Jarret and Litz, 1986; Dewald et al., 1988), and palms (Torres and Tisserat, 1980; McClenaghan and Beauchamp, 1986; Baaziz and Saaidi, 1987; Aljibouri et al., 1988). Based on these studies, it appeared feasible that electrophoretic procedures could become a practical means for date palm cultivar identification, depending on the presence of distinc-

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