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Effects of Ethylene and 1-Methylcyclopropene (1-MCP) on Gene Expression and Activity Profile of α-1,4-Glucan-phosphorylase during Banana Ripening

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Starch phosphorylases are enzymes that can use starch as substrate, and they are supposed to act in both in starch synthesis and degradation. This paper reports the effects of ethylene and 1-methylcyclopropene (1-MCP) on the degradation of starch and phosphorylase activity and gene expression. The results indicate that phosphorylase activity is induced during ripening and that it is associated with the onset of starch degradation. The regulation of banana phosphorylase activity is mainly dependent on gene expression, and the absence of ethylene perception by 1-MCP had a positive effect. However, this effect can be precluded by increased levels of ethylene, both autocatalytic and exogenous.

KEYWORDS: α-1,4-glucan-phosphorylase; banana; starch phosphorylase; 1-methylcyclopropene; fruit ripening

INTRODUCTION

Starch is the main reserve of carbohydrate in plants, and because it is composed of polymers of α-1,4- and α-1,6-linked D-glucose (amylose and amylopectin), several enzymes are argued to be involved in its degradation. Many of them can act on the same substrates and are present in a diversity of isoforms, which was first considered to be as redundancy but now is being recognized as function (*1*).

Starch phosphorylases and α-1,4-glucan-phosphorylase (EC 2.4.1.1) are enzymes that can use starch polymers as substrates, and they are known to play a role in both starch synthesis and degradation. They occur as plastidial (Pho1, plastidial α-1,4-glucan-phosphorylase) and cytosolic (Pho2, cytosolic α-1,4-glucan-phosphorylase) isoforms, which differ not only in localization but in kinetic properties and substrate affinities. Recent studies in pea leaves (*2*) identified a cytosolic heteroglycan probably produced by a cytosolic disproportionating enzyme (DPE2), which would be a suitable substrate for the activity of a cytosolic α-1,4-glucan-phosphorylase. According to the proposed model, the maltose produced from the degradation of starch would be transported to the cytosol and act as a donor of glucosidic units to the heteroglycan acceptor. The resulting high molecular weight glycan could be a source of glucose-1-phosphate by Pho2 activity, thus providing an important metabolite to the cell.

The role played by the plastidial counterpart is still controversial. Although the plastidial α-1,4-glucan-phosphorylase is supposed to act during both starch synthesis and degradation, potato leaves expressing the antisense mRNA for Pho1 did not reveal significant effects on the accumulation of starch in leaves and tubers (*3*). On the other hand, data obtained by Zeeman et al. (*4*) in leaves of mutant *Arabidopsis* indicated a small role for Pho1 in the accumulation of starch during the day and its mobilization at night. However, the mutation induced alterations in the system of tolerance of the plant to the stress provoked by the water deficit, indicating a role for the enzyme in this defense mechanism.

In non-photosynthetic organs such as fruits, the role played by α-1,4-glucan-phosphorylases on starch metabolism is unclear. In bananas, starch can account for almost 20% of the fresh weight of the unripe fruit, and it is converted to soluble sugars in a short period during ripening (*5*). Amylases and α-1,4-glucan-phosphorylases activities can be detected during development and ripening of the fruit. Amylolytic activities during banana ripening were reported by Garcia and Lajolo (*6*), Purgatto et al. (*7*), and Bassinello et al. (*8*), although the effective role of these enzymes in starch degradation is still not clear.

In relation to α-1,4-glucan-phosphorylases, an early work by Arêas and Lajolo (*9*) described a complex pattern of phosphorolytic activity during banana ripening. Later, Mota et al. (*10*) found that α-1,4-glucan-phosphorylase activity seemed to be more related to the fruit development phase, when starch is being synthesized in the pulp of bananas, than to the period when

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full maturity was achieved. They isolated and partially characterized the phosphorylase isoforms, Pho1 and Pho2, and observed that the plastidial isoform presented similar activity levels when assayed for starch synthesis or degradation, indicating that the availability of the suitable substrates would be the preponderant factor in the balance of its activity. In fact, the activity of α-1,4-glucan-phosphorylase followed a complex pattern at the climacteric, not indicating a clear correlation between increased phosphorylase activity and the onset of starch degradation. However, the observed changes in α-1,4-glucan-phosphorylase activity were taken as an indication that modulation of the enzyme would be necessary to the process.

Experiments on the infiltration of indole-acetic acid and gibberellic acid in banana slices allowed to ripen spontaneously did not reveal significant changes in the activity and amount of protein Pho1, although both hormones caused a delay in the degradation of the starch (*10*). These results indicated that these hormones did not have any influence on the regulation of the enzyme. On the other hand, considering that starch degradation is well-correlated to the autocatalytic ethylene production and respiration burst during banana ripening, disturbances in these processes can provide clues about the role played by the activities of several starch-metabolizing enzymes and their regulation. A remarkable effect on banana ripening can be obtained by the application of the ripening trigger ethylene or its antagonist, 1-methylcyclopropene (1-MCP). The conditions resulting from artificially induced or delayed ripening can provide interesting information on the activity and expression of starch-metabolizing enzymes (*11*, *24*). This paper reports the effects of ethylene and 1-MCP on banana ripening and α-1,4-glucan-phosphorylase. We also discuss the consequences on the starch degradation, phosphorylase activity, and gene expression and discuss the possible role of α-1,4-glucan-phosphorylase in the process.

MATERIALS AND METHODS

Plant Material. Preclimacteric bananas (*Musa accuminata* AAA cv. Nanicão) harvested 110 days after anthesis were left to ripen under controlled temperature (20 °C) and humidity (75%). The samples, attached as hands, were placed in chambers (250 L) and treated with 1-MCP (100 nL L^{-1} Ethylbloc, Rohm and Haas, Co.) for 12 h, according to the manufacturer's instructions; ethylene (100 μL L^{-1} in synthetic air) was injected in a gas stream (5 L min^{-1}) for 5 h; fruit not treated constituted the control. Samples taken at different days during ripening were peeled, sliced, frozen in liquid N_2, and stored at −80 °C.

Ethylene and CO_2 Determination. For the ethylene and respiration analysis, bananas were enclosed in 1.5 L jars (three fingers per jar; six jars per treatment). After 1 h, samples of 10 mL for ethylene analysis and 1 mL for CO_2 analysis were taken using a gastight syringe, and the composition of gases was determined by gas chromatography (HP-6890, Agilent Technologies). A flame ionization detector was employed for ethylene analysis and a thermal conductivity detector for CO_2 analysis. For both gases, the column used was HP-Plot Q (30 Mts., i.d. = 0.53 mm, Agilent Technologies); injector and detector temperatures were 250 °C, and an isothermal program was run at 30 °C. Fluxes of helium carrier gas were 1 mL min^{-1} for ethylene and 4 mL min^{-1} for CO_2. The injections were made in pulsed splitless mode for ethylene and in split mode for CO_2 analysis (50:1). Ethylene and CO_2 standards both in synthetic air (Air Liquid Ltd.) were used for calibration curves.

Carbohydrate and Protein Determinations. Starch from frozen samples (100 mg) was solubilized in 3 mL of 0.5 M NaOH. After neutralization with 3 mL of 0.5 M acetic acid, an aliquot was precipitated with 4 mL of 80% ethanol. The precipitated starch was hydrolyzed with amyloglucosidase (EC 3.2.1.3) (28 units/mL) and the resultant glucose determined by the glucose−oxidase (EC 1.1.3.4)−peroxidase (EC 1.11.1.7)−ABTS (2,2′-azinobis[3-ethylbenzthiazoline] sulfonate) system, as described by Cordenunsi and Lajolo (*5*). The protein was estimated according to the Bradford (*12*) method, using bovine serum albumin as standard.

Protein Extraction and Phosphorylase Activity. One part of frozen sample was extracted in 4 parts of 50 mM Hepes−KOH (pH 7.5) containing 20 mM cysteine, 20 mM EDTA (pH 7.0), 1 mM benzamidine, and 1% PVP-40 (polyvinylpyrrolidone 40000). The supernatant obtained after centrifugation at 12000*g* for 10 min was considered to be the crude extract. The α-1,4-glucan-phosphorylase activity was measured in the phosphorolytic direction by the glucose-1-phosphate (Glc-1-P) released from 2% potato soluble starch (*10*). The assay system consisted of 50 mM Tris−maleate buffer (pH 7.5), 50 mM NaF, 2% soluble starch (w/v), enzyme extract, and 500 mM Na_2HPO_4 in a total volume of 500 μL. The reaction was carried out at 30 °C and stopped by heating the mixture in a boiled water bath for 1 min. The released Glc-1-P was determined according to the method of Bergmeyer and Bernt (*13*). Protein was determined according to the method of Bradford (*12*).

Total RNA Extraction, Northern Blotting, and Hybridization. Total RNA was obtained as described by López-Gómez and Gómez-Lim (*14*) and separated according to the method of Sambrook et al. (*15*). For the transfer a nylon membrane (Hybond N+, Amersham Biosciences) was used according to the protocol described by Sambrook et al. (*15*). The Pho1 probe (obtained as described under Probe Development) was labelled with α-[^{32}P]-dCTP, using the Ready-To-Go Labeling Kit (Amersham Biosciences), hybridized at 65 °C, washed, and exposed to an X-ray film at −80 °C (*15*).

Total Protein Extraction and Western Blotting. Total protein was extracted as recommended by Laemmli (*16*) and quantified according to the method of Lowry et al. (*17*) as modified by Peterson (*18*). The fractions were transferred from the gel to a nitrocellulose membrane on 25 mM Trizma and 192 mM glicine (pH 8.3) at constant 30 V for 16 h at 4 °C. To check the equal loading of protein in the lanes, the membrane was stained with Ponceau S, according to the method of Sambrook et al. (*15*). After destaining in water, the membrane was blocked for 1 h using 50 mM Tris-HCl and 150 mM NaCl with 5% skim milk (pH 7.5). After that, it was submitted to the reaction with a specific plastidial α-glucan-phosphorylase antibody (*10*), diluted 1:5000, washed in TBS buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl), incubated with the anti-antibody (conjugated with alkaline phosphatase) in a TBS buffer medium with 5% skim milk (diluted 1:30000), washed again, and developed as described by Sambrook et al. (*15*) using a chromogenic substrate.

Probe Development. The strategy used was the amplification of large fragments of Pho1 cDNA by the rapid amplification of cDNA ends (RACE) technique, using the GeneRacer Kit from Invitrogen, Inc., according to the instructions of the manufacturer. The cDNA from pulp tissue of ripe bananas was obtained using the First Strand cDNA Synthesis Kit (Amersham Biosciences). The PCR reactions were carried out in a thermocycler with specific primers based on a sequence of a Pho1 fragment of 358 base pair (bp) isolated in a cDNA-amplified fragment length polymorphism (cDNA-AFLP) panel, according to the protocol described by Bachem et al. (*19*). The sequences of the primers used in the RACE protocol were 5′-CAA AAC AAA TGG TGT GAC TCC TCG CCG C-3′ (sense) and 5′-GCT TTT TCA TCC AGG AGC ACA CCA GAA G-3′ (reverse). The fragments amplified were visualized in agarose gel electrophoresis (*15*). The fragments were cut from the gel, purified by the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), and cloned using pGEM^R-T Vector Systems (Promega). Transformed colonies were transferred to tubes containing LB−ampicillin medium and incubated for 16 h at 37 °C, and the plasmid was purified by the GFX Micro Plasmid Prep Kit (Amersham Biosciences). Using a Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit and a ReproGel Long Read (Amersham Biosciences) the fragments were sequenced in the ALF Express equipment (Amersham Biosciences). The electrophoresis was carried out at 55 °C for 750 min, and the sequences were analyzed using the Blastx algorithm. A 1256 bp fragment (accession no. AY463025), corresponding to the 3′-end, was obtained with 80% similarity to other

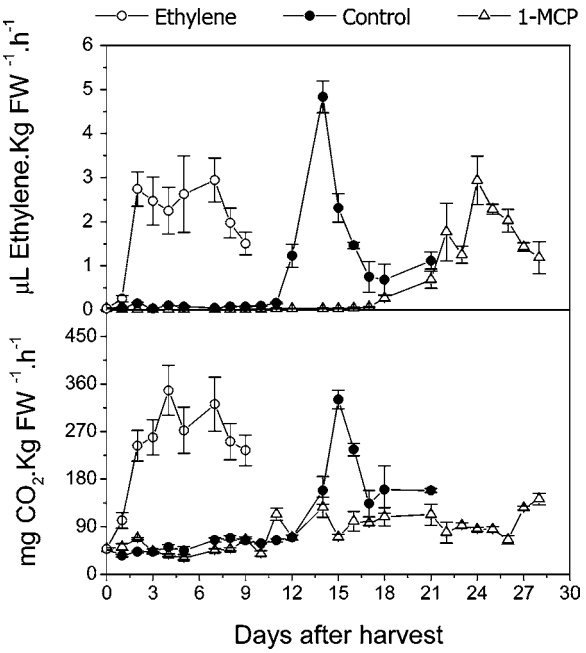

Figure 1. Ethylene production (**A**) and respiration (**B**) during ripening of ethylene-treated (○), 1-MCP-treated (△), and nontreated (control, ●) banana fruit. Each parameter was measured at representative points of the ripening after the treatments. Symbols represent medians and bars, the standard error ($n = 6$).

plastidial α-1,4 glucan-phosphorylases from other plants. This fragment was used as template to generate the radioactive probes for the Northern hybridization procedure.

RESULTS

Data presented in **Figure 1** showed that banana ripening was clearly affected by the treatments. Compared to the control group, the climacteric rise in respiration of fruit exposed to exogenous ethylene was anticipated, whereas those pieces of fruit treated with 1-MCP had no significant increase in respiration, although an ethylene peak could be seen around day 24.

Starch degradation was also affected by the treatments because it proceeded at very different rates for fruit exposed to exogenous ethylene and for those treated with 1-MCP (**Figure 2**). In relation to the activity of starch phosphorylase, a discrete increase was observed during ripening of control fruit, reaching its maximum at the onset of starch degradation. This trend for phosphorylase activity was more evident when fruit ripening was delayed by treatment with 1-MCP, because a more consistent increase could be seen until day 18. For both groups of fruit, enzyme activity was slightly decreased during the late stages of ripening. In the case of fruit that were ripening-induced by ethylene, there was only a marginal diminution in phosphorylase activity for samples containing lower amounts of starch.

Because there are two banana phosphorylase isoforms, which could show different activity profiles during ripening, activity assays were performed after electrophoresis on native gels containing glycogen. Typical gels are presented in **Figure 3**, and according to the densitometric analysis, both Pho1 and Pho2 changed during ripening. The band with low mobility (Pho2), which would correspond to the cytosolic isoform, increased during ripening for all of the conditions tested. In the case of the band corresponding to the plastidial isoform (Pho1), the results for the activity staining indicated an overall increase in activity but different effects of the treatments. Exposure to 1-MCP resulted in induction of Pho1 and its maintenance at higher levels through ripening, whereas exogenous ethylene caused a slight decrease in activity, followed by an apparent recovering of activity at 9 days. On the other hand, Pho1 of the control group peaked at day 4, at the onset of starch degradation, and was slightly decreased thereafter.

To check if the changes in phosphorylase activity were related to the amount of protein, pulp fruit extracts were tested with antiserum against banana Pho1 (**Figure 4**). The densitometric analysis of the blots indicated that enzyme amount increased during fruit ripening and that 1-MCP treatment had a positive effect on banana phosphorylase protein. This increase in protein amount was related to the abundance of phosphorylase transcripts during ripening. According to the densitometric analysis of the normalized Northern blotting presented in **Figure 5**, the amount of phosphorylase mRNA increased during ripening and, similarly to what was observed for the activity and protein, this effect was more evident for those fruit exposed to the ethylene antagonist 1-MCP.

DISCUSSION

The use of exogenous ethylene and its antagonist, 1-MCP, resulted in clearly different groups of fruit. As expected, ethylene

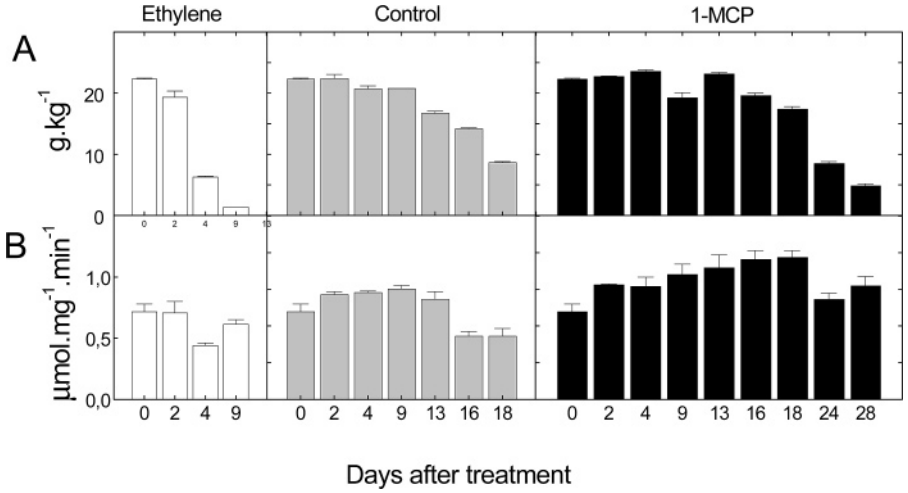

Figure 2. (**A**) Starch degradation and (**B**) total Pho activity, assayed in the phosphorolytic direction during ripening of ethylene-treated (white columns), nontreated (light gray columns), and 1-MCP-treated (black columns) banana fruit. Each parameter was measured at representative points of the ripening after the treatments. Each column represents the median and bars, the standard error ($n = 3$).

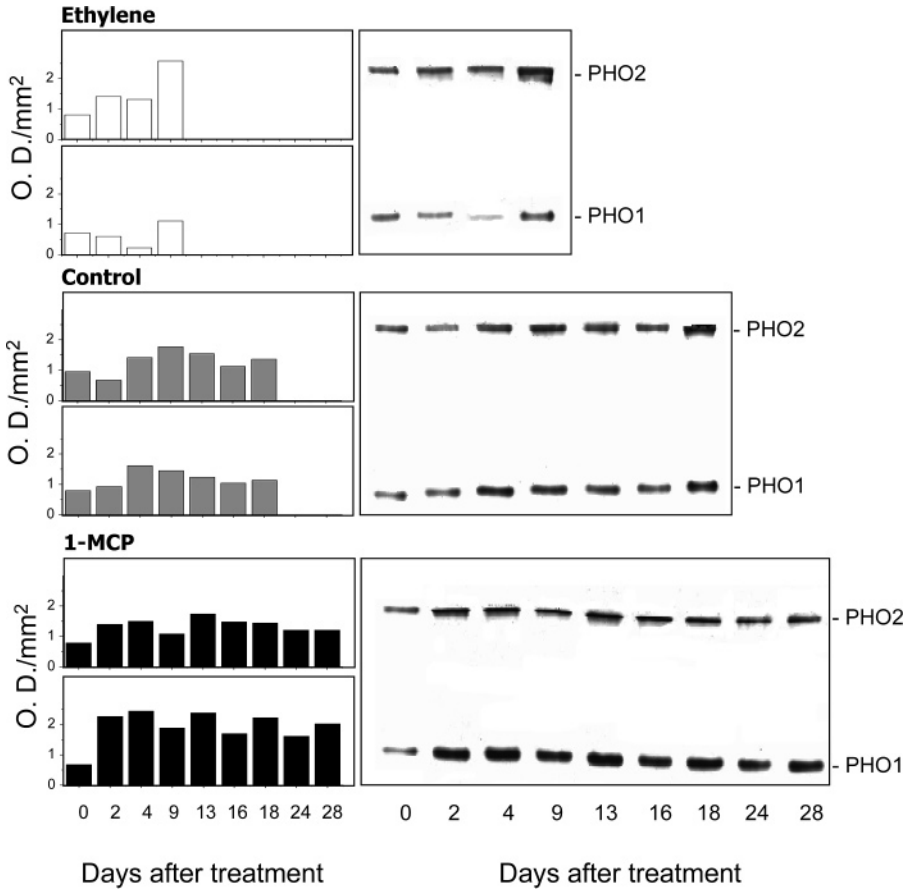

Figure 3. (Left) Densitometric analysis of the activity bands of starch phosphorylase (cytosolic and plastidial as indicated) in polyacrylamide gel. (Right) Gels containing glycogen assayed for starch phosphorylase activity. The total protein was extracted from banana pulps (control, treated with ethylene, and treated with 1-MCP) at several points of the ripening. The upper column graphs, in each group, represent the densitometric analysis of Pho2 (cytosolic) and the lower column graphs, the Pho1 (plastidial). The bands corresponding to the activity of the enzyme were revealed by iodine staining after the incubation of the gel in buffer containing 1% soluble starch and glucose-1-phosphate. The gel was destained in deionized water.

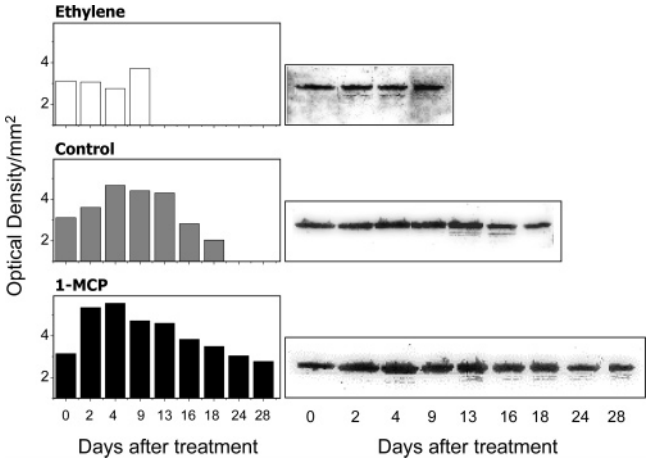

Figure 4. Densitometric analysis (left) and Pho1 Western blot analysis (right) with anti-Pho1 serum, 1:5000 diluted. Total protein extracted from banana pulp of samples treated with ethylene and with 1-MCP. Controls are untreated samples. The amount of total protein loaded in the gel was 50 μg for each sample. Bands of ≈110 kDa. Development with anti-rabbit IgG conjugated with alkaline phosphatase (1:30000) and the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT).

treatment anticipated the events related to ripening, whereas these events were delayed by treatment with 1-MCP. Although the 1-MCP fruit had a recovery of ethylene synthesis capability, which was coincident with the onset of starch degradation for the three groups, there was no typical respiration peak, similar to what was observed in apricot (*20*), broccoli (*21*), tomato (*22*), and apple (*23*).

According to the enzyme activity profiles, banana phosphorylase was also affected by the treatments. Mota et al. (*10*) reported that during ripening of noninduced banana, phosphorylase activity changed in a complex pattern, which was concomitant with the changes in carbohydrate composition. However, there was no clear correlation with the decrease in starch content or with the amount of protein phosphorylase. To have a clearer picture of phosphorylase's role during banana ripening, enzyme activity in solution and gels, along with gene expression and the starch content, was followed during the ripening of fruit samples that were treated under different conditions. The use of the ripening trigger ethylene and its antagonist, 1-MCP, under comparable conditions opened the possibility of evaluating phosphorylase activity under two contrasting situations.

The obtained results indicate that phosphorylase activity is increased during normal ripening and that maximum levels are achieved by the time starch is mobilized. As the polysaccharide is degraded phosphorylase activity is brought to lower levels seen in the unripe fruit. This could be an indication that enzyme activity is demanded during noninduced banana ripening. Because the blockage of ethylene receptors by 1-MCP resulted in increased total activity, and the use of exogenous ethylene precluded any significant increase, it can be suggested that the absence of ethylene perception had a positive effect on phos-

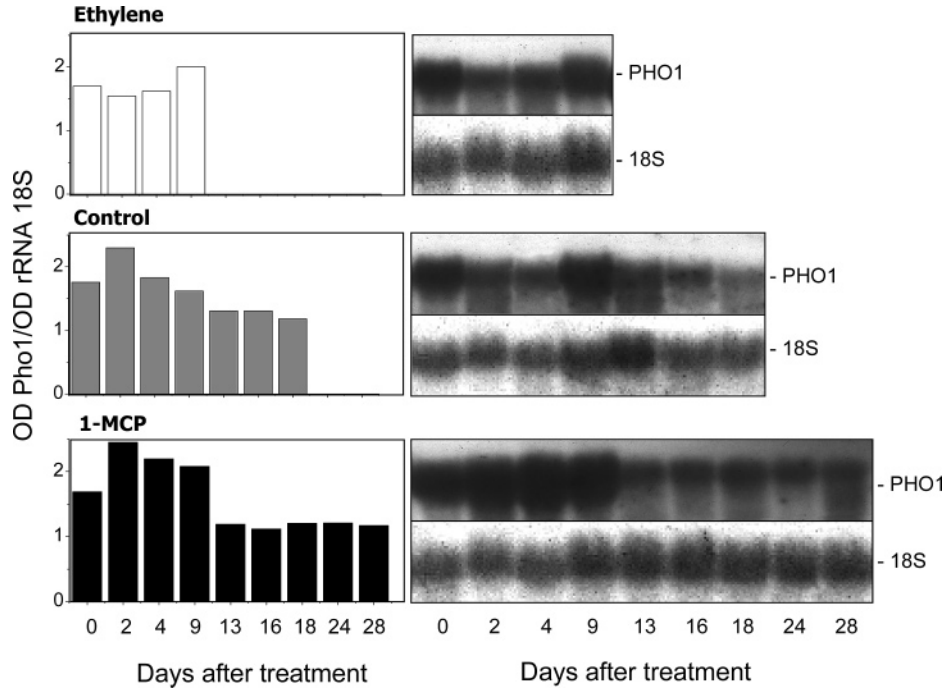

Figure 5. Normalized densitometric analysis (left) and Northern blotting (right) of plastidial starch phosphorylase from total RNA extracted from banana pulp of samples treated with ethylene and with 1-MCP. Controls are untreated samples. The normalized values were obtained by the ratio of the OD from Pho1 bands and the OD from rRNA 18S bands. The expression of ribosome 18S from each sample was the loading control.

phorylase activity. Although the patterns of changes during ripening of 1-MCP-treated fruit were very similar for Pho1 and Pho2, the isoform Pho1 seemed to be more affected by the two treatments. The marked effect of exogenous ethylene on Pho1 activity could be also taken as an indication that this isoform would be more prone to regulation by the hormone. This could also indicate that plastidial phosphorylase would have a greater contribution to the overall phosphorylase activity assayed in bananas and would be in favor of phosphorylase playing a role in starch mobilization during ripening. It would also be possible to speculate that the overeffect caused by 1-MCP on phosphorylase would be a compensatory mechanism for the inhibition of other starch-metabolizing enzymes. In fact, a strong inhibition of β-amylase at transcription and translation levels has been described for 1-MCP-treated bananas (*24*).

Although Mota et al. (*25*) found that the protein amount could not be clearly correlated to the observed changes in phosphorylase activity during ripening, the analysis of banana phosphorylase transcript indicated that changes in enzyme activity are mainly dependent on the modulation of gene expression. Again, the absence of ethylene perception by 1-MCP treatment positively affected the levels of mRNA and protein. In this way a major contribution to the control of enzyme activity would come from the protein level, because the results presented by Mota et al. (*25*) on the kinetic characterization of banana phosphorylases did not indicate allosteric or metabolic regulation of the enzymes. Considering the contrasting effects of exogenous ethylene and 1-MCP treatment on phosphorylase activity and expression, it appears that phosphorylase regulation would be more responsive to changes in basal levels of the hormone than to the autocatalytic burst during the climacteric.

In summary, on the basis of the above-mentioned results, it can be concluded that banana phosphorylase activity is induced during ripening as a consequence of up-regulation of gene expression, which seemed to be responsive to the basal levels of ethylene.

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