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Technical Note

Identification of non-specific alkaline phosphatases in hyphal cells of the fungus *Neurospora crassa* by *in situ* histochemistry

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ABSTRACT. The present study was designed to identify alkaline phosphatases in non-permeabilized hyphal cells of the fungus *Neurospora crassa* by staining these enzymatic activities with a modified azo dye coupling method. Our strategy allowed the identification of three non-specific alkaline phosphatase activities, one of them possibly being a novel putative enzyme, which is not responsive to either Mg^{2+} or EDTA. Another alkaline phosphatase activity, whose location in the hyphal cell is regulated by phosphate, is stimulated by Mg^{2+}, inhibited by EDTA, and somehow dependent on the expression of the *pho*-2^{+}-encoded Pi-repressible alkaline phosphatase.

Key words: Alkaline phosphatase, Gene regulation, *Neurospora crassa*, Phosphate sensing

The phosphate (Pi)-repressible alkaline phosphatase (APase), in addition to other related enzymes synthesized by the fungus *Neurospora crassa* under Pi starvation, belongs to a group of enzymes that hydrolyze phosphate esters to provide the cell with Pi (Metzenberg, 1979). The *pho*-2 gene, which encodes the Pi-repressible APase PHO-2, is expressed regardless of growth medium pH, but the active enzyme is secreted into the growth medium only at alkaline pH (Nahas et al., 1982; Nozawa et al., 2002). Also, the PHO-2 APase retained by the mycelium or secreted into the growth medium by the 74A strain is activated by EDTA, but is not affected by Mg^{2+} (Lehman and Metzenberg, 1976). Furthermore, the PHO-2 APase is inactive in the mutant strain *pho*-2A, which provides an important control for assaying other APases. Thus, the present study was designed to identify APases in non-permeabilized hyphal cells of the mold *N. crassa* by staining these activities with Fast Red TR (Sigma), and observing them as a brown stain under light microscopy, i.e., our aim was to identify APases that are transported to the plasma membrane or to the periplasmic space of the hyphal cells. Our strategy revealed the presence of at least a novel putative APase in *N. crassa*, which is not responsive to either Mg^{2+} or EDTA.

The strains St. L. 74A (wild type) and *pho*-2A (FGSC 3061) of *N. crassa* used throughout this study are available from the Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, MI, USA). The *pho*-2A strain was identified as carrying a mutation in the structural gene for the Pi-repressible APase (Groteluesch en et al., 1994). A spore suspension from each fungal strain (about 5 x 10^7 cells) was grown for 24 h and 48 h without shaking, at 30°C, in 50 mL high- (10 mM) or low- (50 µM) Pi medium adjusted to pH 7.8 (buffered with 50 mM Tris-HCl) (Nahas and Rossi, 1984), supplemented with 44 mM sucrose as the carbon source (Crocken and Nyc, 1963). The APase activities were visualized in non-permeabilized hyphae of the fungus *N. crassa* by a modified azo dye coupling method using fast red TR salt (1,5-naphthalenedisulfonate salt) (Sigma) in the presence of α-naphthyl acid phosphate (Dorn, 1965; Fiskin et al., 1986; Tisserant et al., 1993; van Aarle et al., 2001). The staining mixture consisted of 2.5 mM α-naphthyl acid phosphate, 5 mM fast red TR, and 0.3 M Tris-HCl buffer, pH 8.9, supplemented with 1 mM Mg^{2+} or with 1 mM EDTA. Freshly collected hyphae were rinsed with 0.3 M Tris-HCl buffer, pH 8.9, flooded with the staining mixture for 15 min at room temperature, rinsed with distilled water, flooded with 1% methyl green for 2 min, rinsed with distilled water, mounted with glycerin under coverslips, and examined with a light photomicroscope.

The staining procedure used in the present study allowed the identification of three distinct non-specific APase activities in non-permeabilized hyphal cells of the fungus *N. crassa*. The PHO-2 APase, which is activated by EDTA, is readily observed in hyphal cells of the wild-type strain grown for 24 h under Pi restriction, an enzyme inactive in the *pho-2A* mutant strain (Figure 1). It can also be observed in Figure 1 that this enzyme is, as previously described (Han et al., 1987, 1992) largely secreted into the growth medium during the first 48 h of cultivation. The second enzymatic activity identified appeared to be a novel enzyme since its synthesis, which is better observed in hyphal cells of the *pho-2A* strain grown for 48 h, occurred regardless of the levels of Pi in the growth medium. Also, its enzymatic activity did not respond to Mg^{2+} or EDTA (Figure 1). The third APase activity identified in hyphal cells of the wild-type strain grown for 48 h under Pi restriction, which is probably located in the periplasmic space, was stimulated by Mg^{2+} and inhibited by EDTA, i.e., having properties shown by the constitutive APase (Morales et al., 2000). Nevertheless, the constitutive APase was identified in permeabil-

ized hyphal cells and characterized as an intracellular enzyme (Basabe et al., 1979), whereas the enzymatic activity revealed in the present study (Figure 1) is apparently located in the periplasmic space of the hyphal cell, an event regulated by Pi (Figure 1). Furthermore, its activity is somehow dependent on the expression of the PHO-2 APase (Figure 1). It is worth noting that several functions other than that involved in the hydrolysis of phosphate esters to provide the cell with Pi have been proposed for the PHO-2-like APases, including their role as transferases in transphosphorylations, in transmembrane transport of Pi, and in the regulation of the *pho*-3^{+}-encoded Pi-repressible acid phosphatase in *N. crassa* (Han et al., 1992; Tisserant et al., 1993.

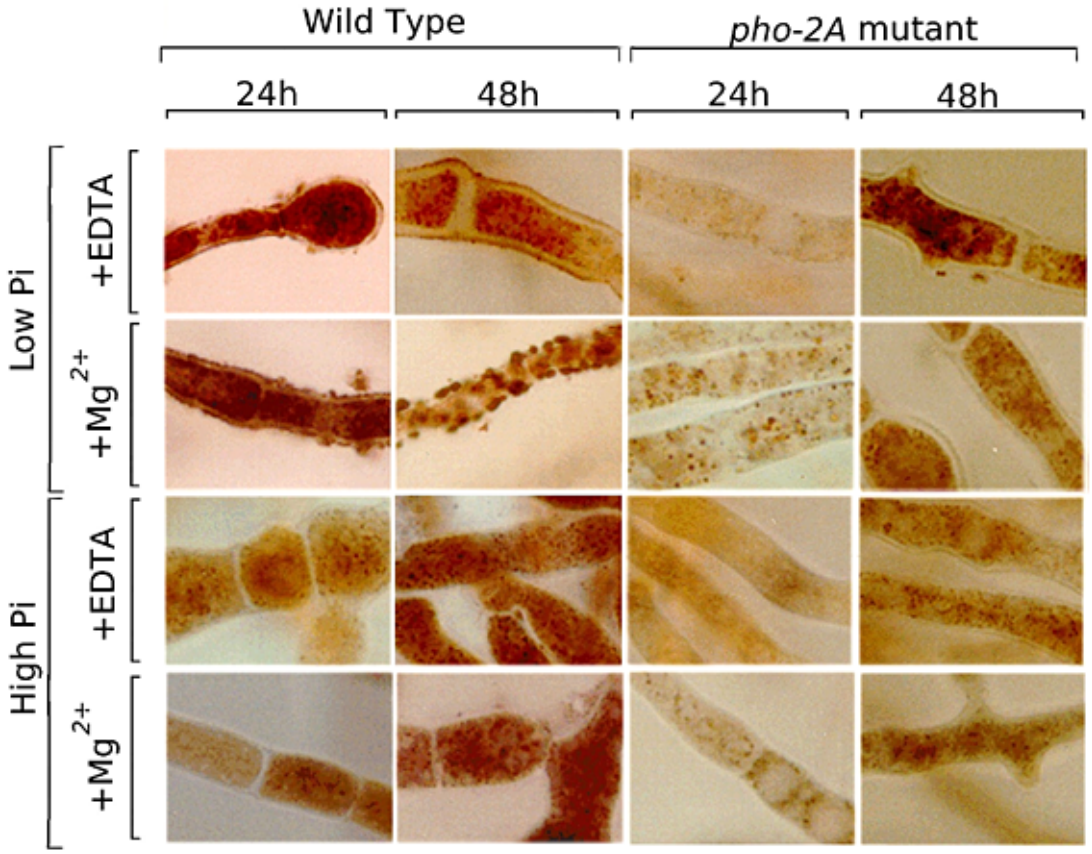

Figure 1. Light micrographs of hyphal cells of the fungus *Neurospora crassa* stained for non-specific alkaline phosphatase activities in the presence of 1 mM EDTA (+EDTA) or 1 mM Mg^{2+} (+Mg^{2+}). The wild-type (74A) and *pho*-2A mutant strains were grown in low- and high-Pi medium (50 µM and 10 mM Pi final concentrations, respectively) for 24 and 48 h at 30°C, pH 7.8.

In conclusion, the easily performable histochemical procedure used in the present study allowed the identification of three non-specific APase activities in non-permeabilized hyphal cells of the mold *N. crassa* grown at pH 7.8, one of them possibly being a novel putative APase. Nevertheless, it is clear that additional efforts are necessary to characterize all the putative non-specific APases revealed by the *N. crassa* genome sequencing project (www.fgsc.net).

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