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Detection of a Histidine Kinase mRNA in Extraradical Mycelium of *Pisolithus tinctorius* Induced by the Plant Metabolites

[1]Aseneth Herrera-Martínez, [1]Roberto Ruiz-Medrano, [2]María Valdés and [1]Beatriz Xoconostle-Cázares
[1]Departamento de Biotecnología y Bioingeniería,
Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional,
Av. IPN 2508, San Pedro Zacatenco, 07360, México, D.F.
[2]Escuela Nacional de Ciencias Biológicas, IPN, Plan de Ayala y Carpio, 11340, México, D.F.

Abstract: The aim of this study was to test the effect of acetosyringone (AS) on the accumulation of the histidine kinase coding mRNA, using *in vitro* propagated *Pisolithus tinctorius*. In the precontact phase of the ectomycorrhizal symbiosis, it is hypothesized that the plants releases chemicals, which are sensed by the fungal mycelium and in turn trigger the start of the symbiosis. The nature of such molecules is largely unknown; however, plant metabolite and their structural analogues have been widely used to induce infection in different microbe-plant interactions. A histidine kinase in *Agrobacterium tumefaciens* was activated by AS in the first step during the infection of dicotyledonous plants. A conserved gene fragment from the ectomycorrhizal fungus *Pisolithus tinctorius* was cloned using degenerate primers of conserved regions characteristic of the histidine kinase gene family. Then, the accumulation of this transcript in fungi incubated with AS was analyzed. RT-PCR and *in situ* hybridization suggest that this mRNA is synthesized in the presence of this plant analog in AS-treated mycelia. The findings presented here suggest a role of a histidine kinase involved in the early stages of ectomycorrhizal differentiation.

Key words: mRNA *in situ* hybridization, ectomycorrhiza, signal transduction, acetosyringone

INTRODUCTION

Ectomycorrhizal fungi associate with gymnosperms and angiosperm and are widespread in both temperate and boreal forests (Marx, 1977). The basidiomycete *Pisolithus tinctorius* has been used as a model system for ectomycorrhizal symbiosis (Smith and Read, 1997). It is assumed that the ectomycorrizal symbiosis results from the activation of developmental programs in both symbionts, which undergo morphological adaptations. These modifications allow the mobilization and transport of mineral nutrients by the fungus, from soil to the root, while the plant provides photoassimilates to the fungus (Podila *et al*., 2002; Akiyama *et al*., 2005). There is a limitation in basic knowledge of earliest steps at the molecular level during the establishment of the symbiosis, although it is clear that it relies on the exchange and perception of chemical signals, by specific receptors, between both prospective partners (Barker *et al*., 1998; Barker and Tagu, 2000). However, the receptors for such compounds have not been identified to date. Diverse signaling pathways are involved in the adaptation to different environmental conditions. Furthermore, some of these pathways partially overlap with those involved in the recognition of the host by bacterial and fungal pathogens, as is the case of two-component systems (Brencic and Winans, 2005; Jones *et al*., 2007). The initial step in these systems is a histidine kinase that phosphorylates an effector protein. The human pathogen *Candida albicans* possesses a two-component system that responds to different stress conditions and is involved in pathogenicity, in response to signals derived from its host. These transduction pathways could be involved in the recognition process between symbionts as well.

In this study, the cloning and accumulation of histidine kinase transcript of a conserved fragment of the histidine kinase-encoding gene from the fungus *Pisolithus tinctorius* isolated from Central Mexico is reported as induced by the plant elicitor acetosyringone.

MATERIALS AND METHODS

In the present study, the strain *Pisolithus tinctorius* isolated from semiarid region in Queretaro, Mexico was employed (Valdés, 1985; Rodriguez-Tovar *et al*., 2005).

Corresponding Author: Beatriz Xoconostle-Cázares, Biotecnologia, CINVESTAV IPN,Av. IPN 2508, San Pedro Zacatenco, 07360, Mexico, D.F.

General molecular biology procedures were carried out essentially as described by Sambrook and Russell (2001). Based on the comparison of fungal histidine kinases, degenerate primers are composed of forward 5'-CAYGARATNMGNACNCCNATNAAYGGNAT-3' and reverse 5'-GTRAAYTTNACNGCRTTNCCNACNARRTT-3'. A fragment of 377 bp product was amplified using *P. tinctorius* cDNA, synthesized from total RNA extracted from acetosyringone-treated mycelia, cloned into the pCRII TOPO Dual vector (Invitrogen) and sequenced. In order to test its accumulation in the presence of AS, a linear RT-PCR was performed as follows: total RNA from saprophytic fungus induced with 140 mM of AS and control was isolated. Incubation was done with *DNase* I and then used to synthesize cDNA using oligo d(T).

After first strand synthesis, DNA was normalized using the amplification of the 28S rRNA using the primers, forward 5'-ATCTTTCCCTCACGGGACTTGTTC-3' and reverse 5'-GGTAGGAACACCCGCTGAACTTA-3' and then employed to amplify the histidine kinase cDNA.

In situ mRNA hybridization was performed as described by Ruiz-Medrano *et al.* (1999) with the following modifications. Fungal tissue was fixed in FAA buffer for 2 h and incubated with 2 μg mL^{-1} proteinase K for 10 min. Cells were washed twice with 1X TBS (Sambrook and Russell, 2001) and preincubated in hybridization buffer. Sense and antisense histidine kinase RNA probes were synthesized labeled with UTP-digoxigenin by *in vitro* transcription using a commercial system following the manufacturer's recommendations (Maxiscript, Ambion Austin TX).

RESULTS AND DISCUSSION

Identification of a conserved fragment of the Pisolithus tinctorius His-kinase gene His-kinase mRNA accumulates in response to AS incubation.

The amplification of the His-kinase fragment in cDNA synthesized from AS-treated cells was observed. A band of the expected size is observed in AS-treated mycelia but not in untreated control cells (Fig. 1). The amplified DNA was cloned, sequenced and compared with the extant databases. The highest homology at the amino acid level (98%) was obtained with a sequence from *Laccaria bicolor*, whose genome sequence has been recently obtained and annotated (Martin *et al*., 2008).

His-kinase mRNA locates in extraradical mycelia: In order to detect the presence of mRNA *in situ*, AS-induced saprophytic mycelia was collected and processed as described by Ruiz-Medrano *et al.* (1999). AS-treated mycelia displayed an intense purple signal only with antisense probe (Fig. 2a, b). Histidine kinase expression is

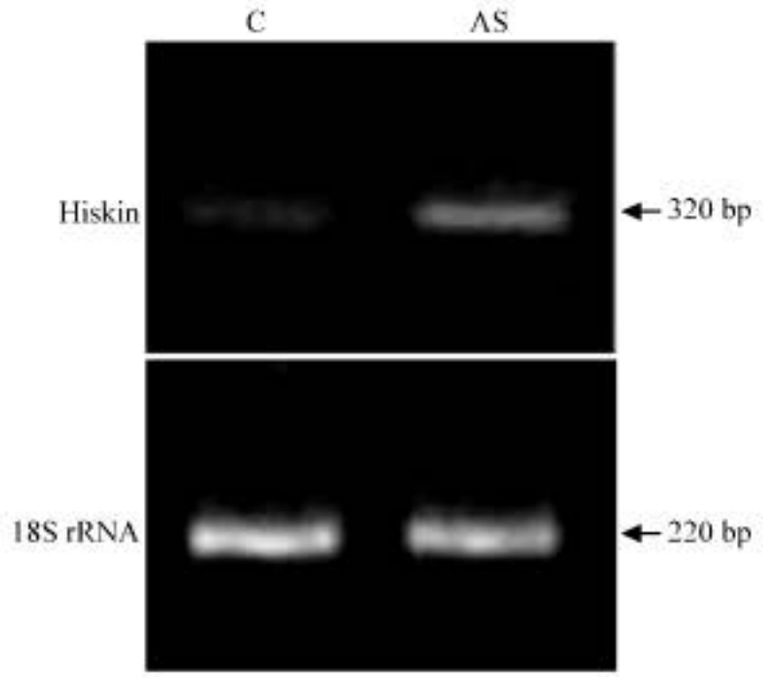

Fig. 1: *Histidine kinase* mRNA accumulates when fungal cells are incubated with acetosyringone. Linear RT-PCR was assayed using total RNA extracted from previously incubated fungal cells with the indicated treatment. Specific primers used for *Histidine kinase* and 28S are indicated in material and methods

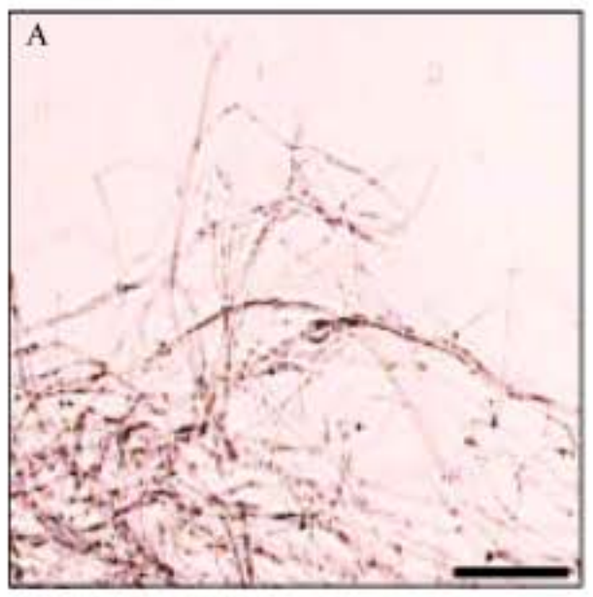

Fig. 2: *In situ* mRNA hybridization of histidine kinase in fungal mycelium. Panel (A) Saprophytic mycelium treated with AS; (B) untreated, control mycelium. Both samples were assayed with UTP-labeled sense and antisense RNA. Bar in (A) is 50 μm and is common to (B) to (D)

regulated at both the transcriptional and posttranslational levels (Grefen and Harter, 2004; Calera *et al*., 2000); in the first case possibly through a positive feedback mechanism and in the latter, conceivably through conformational changes due to binding to specific ligands and by phosphorylation of a conserved histidine residue. Regardless, the induction of histidine kinases result in the activation of responses to a variety of stimuli, as has been described in plant and fungi (Lee *et al*., 1996; Klumpp and Krieglstein, 2002).

In the study work the identification of a histidine kinase gene from the ectomycorrhizal fungus *P. tinctorius*, of which a fragment corresponding to the most conserved domain from fungal histidine kinases is reported.

In conclusion, the findings present here support the notion that the histidine kinase transcript accumulated when *in vitro* propagated mycelia were exposed to an analogue of a plant cell wall metabolite, acetosyringone. Additional experimental approach is necessary to elucidate the role of *Pthik1* in the ectomycorrhizal symbiosis.

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