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The Amazing Role of the Group III of Histidine Kinases in Plant Pathogenic Fungi, an Insight to Fungicide Resistance

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ABSTRACT

This study is focused to review some of the most exciting discoveries regarding how plant pathogenic fungi use the histidine-kinase phosphorelay to coordinate distinct events of their life; such is the case of osmotic adjustment, cell cycle regulation, virulence, cell wall assembly, sensing and response to environmental changes and finally, how pathogenic fungi acquires fungicide resistance against dicarboxiimide and phenylpyrroles. Particular emphasis is given to the group III of histidine-kinases, also known as the NIK1 class, because experimental evidence through gene sequencing, mutant isolation and gene knock outs is showing that in plant pathogenic fungi, the HK group III is the main responsible for the acquisition of resistance to some important fungicides. These finding support and suggests that fundamental changes should be considered in the strategies that are currently being used to control important plant pathogens like *Botrytis cinerea*, *Cochliobolus heterostrophus, Alternaria brassicicola* and *Magnaporthe grisea*.

Key words: Pathogenic fungi, histidine-kinase, fungicide

INTRODUCTION

The living organisms actively interact with their surrounding environment and modulate their physiology to maintain cellular homeostasis. This process is coordinated via diverse signaling pathways and involves several signaling components, including sensors/receptors, kinases and transcription factors (Chauhan and Calderone, 2008).

Protein phosphorylation is a postranslational modification by covalent addition of phosphate groups to serine and threonine amino acids and to a lesser extent to tyrosine and histidine (Klumpp and Krieglstein, 2002). These modifications are carried out by enzymes known as serine or serine/threonine kinases, tyrosine kinases and histidine kinases. All of them transfer the γ-phosphate from ATP to their substrates. Phosphorylation of serine, threonine or tyrosine residues results in the formation of phosphoester linkage, while phosphorylation of the histidine residue occurs on 1- or 3-nitrogen atoms of its imidazole ring, then producing a phosphoramidate bond (Fig. 1). This linkage is highly unstable making phosphohistidine the most labile of phosphoamino

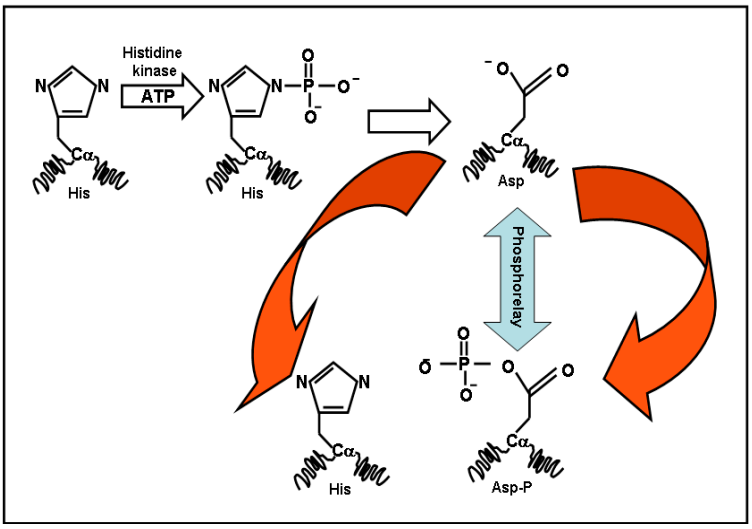

Fig. 1: The histidine phosphorylation and the histidine-aspartate phosphorelay in protein signaling. The nitrogen 1 or 3 of the imidazole ring from an histidine located in a conserved motif, is the initial target for histidine kinases. After transient histidine phosphorylation, the phosphate group is transferred in a reversible reaction to a conserved aspartate placed into a receiver domain, which can be present in the same protein or in a different protein; adapted from Thomason and Kay (2000)

acids (Thomason and Kay, 2000); phosphohistidine is unstable under acidic conditions and has a relatively high ΔG of hydrolysis, such characteristics prevent that many of the techniques applied to study serine/threonine and tyrosine kinases be used to study histidine kinases (Thomason and Kay, 2000).

Protein phosphorylation is one of the paths to regulate and coordinate the signal transduction for the control of responses to environmental changes, mating, osmosensing, cell defense, nuclear rearrangements. These facilitate gene transcription and in addition response to growth factors (Islas-Flores *et al*., 1998, 2000). Thus, protein phosphorylation is a largely important event; both prokaryotic and eukaryotic have organized their fundamental metabolic pathways in sequential and coordinated reactions that are tightly regulated through protein phosphorylation, such is the case of MAP-kinase cascade or the cell cycle coordination (Islas-Flores *et al*., 2000).

In fungi, several signaling cascades have been involved in stress response and adaptation. Among them, the HOG (High-Osmolarity Glycerol response) pathway is one of the best characterized. The HOG signaling pathway was firstly identified as a crucial component playing a pivotal role in maintaining water balance in hyper-and hypo-osmotic conditions in the budding yeast *Saccharomyces cerevisiae*. In fungi, the HOG pathway is also a major controller governing cellular responses to diverse external stimuli including UV irradiation, oxidative stress, heavy metal stress and high temperature stress. Furthermore, in pathogenic fungi, the HOG pathway is involved in growth regulation, morphology, differentiation, virulence, melanin synthesis and polymorphic transitions. All of the last events are regulated through protein phosphorylation, an event that involves the addition/removal of phosphate groups from protein by kinase/phosphatase enzymes (Wolanin *et al*., 2002).

In fungi, histidine phosphorylation plays essential roles in the regulation of key pathways that govern fungal life. This review is focused on the importance of fungal histidine-kinases for fungal survival and particularly to highlight the characteristics of the members of the group III histidine kinases. This group of kinases was first related with regulation of the osmotic adjustment and virulence, but more recently it has been shown that the members of this family are related to the control of the HOG pathway and with the acquisition of fungicide (dicarboxiimide and phenylpyrrole) resistance (Yoshimi *et al*., 2004). This family of kinases is also known as NIK1 group.

WHAT IS HIS-ASP PHOSPHORELAY?

Until 1993 it was thought that the histidine phosphorylation-based signaling mechanism was restricted to prokaryotes (Parkinson, 1993). This idea changed when the groups of Chang *et al*. (1993) and Maeda *et al*. (1994) discovered that it is critical for environmental responses in yeast. Several years later, similar findings were observed in plant cells (Desikan *et al*., 2008). With the first discoveries it was established that the histidine-aspartate phosphorelay consists of a series of signal transduction proteins that alternately posses a site of histidine or aspartate phosphorylation, or both (Fig. 2). His-Asp phosphorelay pathways comprise up three types of signaling elements.

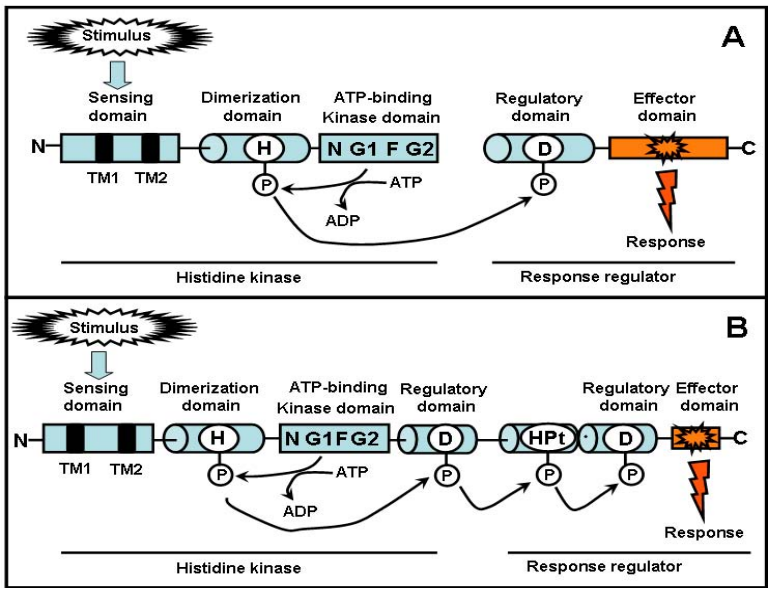

Fig. 2: Schematic diagram of simple (A) or hybrid phosphorelay (B) in the His-Asp two components systems. External signals are sensed by the sensor domain and triggers phosphorylation of a conserved histidine residue (H). The phosphoryl group is transferred to a conserved aspartic acid residue (D) in the regulatory domain (D), activating the effector domain and result in an output response. In a hybrid two component system protein contains both, the histidine kinase and regulatory domains and generally requires additional rounds of phosphorelay through an histidine phosphotransfer (HPt) domain and a second regulatory domain. The HPt domain can be part of the hybrid HK protein or separate protein. Most eukaryotic and fungal HKs are hybrids; adapted from Catlett *et al*. (2003)

First, an histidine protein kinase (HK), which contains a site of histidine phosphorylation and acts as a transmitter. The HK works as a dimer, transphophorylating its partner subunit, using the γ-phosphoryl group of ATP (Bilwes *et al*., 1999). Subsequently the γ-phosphoryl group from HK is transferred to a response regulator protein that has a receiver domain containing a conserved site of aspartate phosphorylation. Aspartate phosphorylation in the response regulator acts as a biochemical switch to control output activity, thus transmuting the original signal (Parkinson, 1993). A third module, the histidine phosphotransfer (HPt) domain allows different receiver domains to communicate with each other in more complex phosphorelay (Wolanin *et al*., 2002). In many cases, the histidine kinases themselves are transmembrane receptors that have an N-terminal sensing domain; in other cases, the histidine kinase is in the cytosol and it has a separate sensing module in the membrane to which it may couple.

THE HISTIDINE-ASPARTATE PHOSPHORELAY PATHWAY IN EUKARYOTES, AN UNEXPECTED BUT EXCITING DISCOVERY

As mentioned above until recently it was thought that His-Asp phosphorelay pathways were absent in the animal kingdown. Today, genome projects and the development of new highly specific and sensitive biochemical assays are revealing that His-Asp phosphorelays are undoubtedly present as multigene families in prokaryotes (Kohanski and Collins, 2008), lower eukaryotes (Chauhan *et al*., 2007), plants (Cho and Yoo, 2007; Desikan *et al*., 2008) and might be present in mitochondria from mammalian cells (Besant and Attwood, 2005). Presence of this system in mammalian cells would prevent targeting His-Asp signaling to arrest bacterial and fungal mammalian pathogens as has been largely proposed (Santos and Shiozaki, 2001; Chauhan and Calderone, 2008). However, a major challenge is to understand first how the His-Asp signal transduction systems forms integrated networks with the more familiar signaling mechanisms (serine, serine-threonine or tyrosine kinases) also present in eukaryotic cells.

ARE HISTIDINE KINASES AND SERINE, SERINE/THREONINE AND TYROSINE KINASES RELATED?

Histidine kinases maintain scarce relationship with the serine, serine/threonine or tyrosine kinases, although all they catalyze the γ-phospho transfer from ATP to specific amino acid residues (Hernández-Sotomayor and Islas-Flores, 2002). Analysis at the level of nucleotide sequence and amino acid sequence also confirm the limited similarity among them. Particular comparison of kinase activity between the histidine and the others is largely complicated because histidine kinases have a narrow spectrum of substrates, including themselves, in comparison with the possible substrates for serine, serine/threonine or tyrosine kinases (Islas-Flores *et al*., 2000). Additionally, the phosphorylated histidine and aspartate residues are unstable, which has prevented the production of anti-phosphohistidine or anti-phosphoaspartate antibodies, valuable analytical existing tools for phospho-serine, phospho-serine/phospho-threonine or phospho-tyrosine (Klumpp and Krieglstein, 2002).

THE BEGINNING OF PHOSPHORELAY HIST-ASP ACTIVATION PATHWAYS, A SINGLE OR A COUPLE?

The prokaryotic and eukaryotic phosphorelay system occurs essentially in two classes, the single version prevalent in bacteria and usually called two-component system. Here a histidine kinase and a response regulator directly relay phosphate to one another. An example is the EnvZ-OmpR

pathway from *E. coli* (Appleby *et al*., 1996). Eukaryotes more commonly use a histidine kinase containing in the same polypeptide a receiver domain; this is known as a hybrid kinase. Phosphate is first transferred from the histidine residue in the transmitter to the aspartate residue on the receiver domain, then to a histidine residue on the histidine phosphotransfer domain (on the same protein or in a separate protein). Finally the phosphate is relayed from the HPt domain to the receiver domain of a downstream response regulator protein, which results in the output response (Kohanski and Collins, 2008).

THE HIS-ASP PHOSPHORELAY PATHWAY IN FUNGI

Fungi (both pathogenic and non pathogenic) strongly influence the behavior and health of plants and animals where they live and additionally they must face and overcome changes in the environmental or host and rapidly adjust their metabolism (Lizama-Uc *et al*., 2007). Fungi use two-component systems to initiate intracellular signaling pathways involving diverse signaling elements such as Mitogen-Activated Protein Kinases (MAPK) and cyclic nucleotides (West and Stock, 2001). These signal transduction pathways act as columns that channel the stimuli and then transduce it into the language of cells. One of the best-studied signaling cascades, the High Osmolarity Glycerol (HOG) cascade, which mediates the osmotic and oxidative stress responses of the budding and fission yeast (Hohmann, 2002), act as a histidine kinase phosphorelay pathway, thus constituting an excellent model to analyze the regulation of histidine kinase enzymes and how the modification of such enzymes influences the behavior of pathogenic and non-pathogenic fungi.

The HOG signaling pathway is evolutionary conserved in fungi but it also clearly exhibits particular characteristics in the model yeasts *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast). Both of them are used to compare the HOG pathway of fungal pathogens but *S. pombe* seems to be a more appropriate model because it harbors multiple hybrid sensor kinases with diverse subdomains. Additionally, HOG in *S. pombe* not only responds to multiple external stimuli, including osmotic shock, oxidative stress, heat shock and UV irradiation, but also controls the differentiation and morphology of cells. Similar pleiotropic roles of the HOG pathway have also been described in fungal pathogens. Finally, in *S. pombe* the Wak1-Wis1-Sty1 pathway is mainly controlled by the two-component system, as in pathogenic fungi (Hohmann, 2002).

In *Saccharomyces cerevisiae*, hyperosmotic conditions lead to the dephosphorylation of the transmembrane HK (Sln1) that negatively regulates the MAPK cascade, via the phosphorelay elements Ypd1 and Ssk1. The MAPK cascade works by sequential phosphorylation of a MAPKKK (Ssk2/Ssk22), a MAPKK (Pbs2) and the MAPK Hog1, which migrates to the nucleus to modulate the activity of several transcription factors. Regulation of the HOG pathway also involves another osmosensing branch, the Sho1 route, which is partially functionally redundant with the Sln1 branch and has several protein kinases and phosphatases in common with other MAPK pathways. This enables cross-talk of signaling cascades regulating different cellular functions, such as adaptation to medium osmolarity, cell integrity control, sterile vegetative growth, resistance to elevated temperatures and oxidative stress (Alonso-Monge *et al*., 2001).

Pathogenic fungi have also developed distinct and divergent features in the function and regulation of the HOG pathway with respect to the fission yeast. Some pathogenic fungi, such as *Aspergillus fumigatus* and *Criptococcus neoformans*, contain more hybrid sensor kinases than fission yeast. This may reflect adaptation to diversity in environment and biological niches because these pathogens survive and proliferate in both natural environments and human hosts

(Bahn, 2008). Although, there are diverse and multiple hybrid sensor kinases in pathogenic fungi, the comprehensive and integrative understanding of the role of all hybrid sensor kinases is far from completion.

IMPORTANCE OF THE HIS-ASP PHOSPHORELAY AND THE GROUP III OF HISTIDINE KINASES IN PHYTOPATHOGENIC ASCOMYCETES

In filamentous fungi, homologues of individual elements of the budding yeast HOG1 and fission yeast Sty1 pathway have been identified by functional and *in silico* analysis (Kojima *et al*., 2004). *Neurospora crassa* OS-2 is homologous to budding yeast HOG1 (Zhang *et al*., 2002) and is regulated by the MAPKK OS-5 and the MAPKKK OS-4 (Fujimura *et al*., 2000). Potential orthologs of Sln1, Sho1, Ypd1, Ssk1 and Ste11 have been found in the genome of *Neurospora crassa*. However, the phosphorelay mainly responsible for osmosensing, OS-1 (also known as *NIK1*), is not an ortholog of *Sln1* (Catlett *et al*., 2003).

A genome search for the two-component signal genes in the phytopathogenic *Cochliobolus heterostrophus*, *Gibberella moniliformis* and *Botrytis cinerea* revealed the existence of an extensive family of histidine kinase genes that can be grouped into eleven classes (Fig. 3). The NIK1/OS-1 class III group has representatives in all the studied euascomycete species. The NIK1/OS-1 class III group polypeptides (i.e., *N. crassa* Os-1p (also known as Nik1p), *B. fuckeliana* Daf1p (also known as BcOs1p) and *C. heterostrophus* DIC1p (also known as ChNik1p) differ significantly from members of the Sln1/TcsB HK class. In addition to the usual phosphoacceptor ATP-binding and RR domains, NIK1 has a N-terminal region that contains several repeats of the HAMP domain (Catlett *et al*., 2003), which is a domain of currently unknown function. The class III group also lack of transmembrane domains present in the Sln1 HK class, implying that the NIK1 is a cytoplasmic protein (Fig. 4).

Histidine kinases are large proteins with >1300 amino acids and contains conserved H-, N-, G1- and G2-boxes. The H-motif is where the His residue of the phosphorelay is located and the

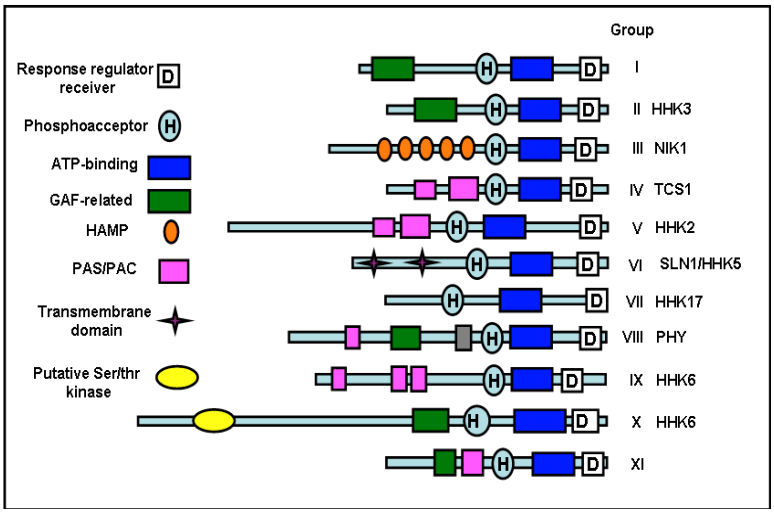

Fig. 3: Diagramatic representation of histidine kinase structure and domain organization in the different groups comprising the histidine kinase family. Each diagram is drawn at scale considering the protein size; adapted from Catlett *et al*. (2003)

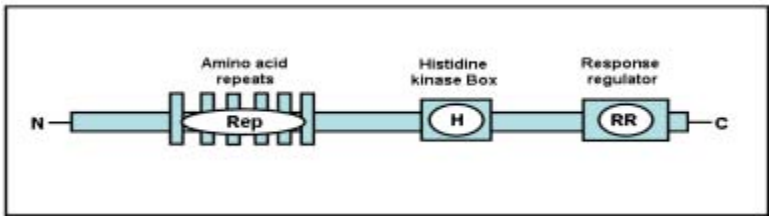

Fig. 4: Schematic representation of the structure and domain organization in NIK1 members. The Nik1 has six amino acid repeats near to the amino terminal, a histidine kinase domain and the response regulator domain; modified from Kruppa *et al.* (2004)

N-, G1-, F- and G2- motifs define the nucleotide binding cleft of histidine kinases, near to the C-terminus, lies the receiver domain (response regulator) where is located the Asp residue of the phosphorelay (Wolanin *et al.*, 2002). Fungal histidine kinases have a unique N-terminal region consisting of HAMP domain repeats. This region contains a total of 11 coiled-coils and probably is essential for the proper functioning of the fungal histidine kinase. Although, the precise function of this domain is still elusive, mutations in the NIK1 HAMP repeated region result in severe osmosensitivity and dicarboximide resistance in *N. crassa* (Miller *et al.*, 2002). In the case of class III histidine kinases from filamentous fungi it has only six 90-amino-acid repeats motif near the N-terminus, putatively forming a coiled-coil domain (Catlett *et al.*, 2003). Proteins belonging to this group enlist CaNIK1p (*C. albicans*), NcNIK-1/Os-1p (*N. crassa*), BcBOS1p (*B. cinerea*) and BmHK1p (*C. heterostrophus*).

Genetic studies on NIK1 orthologs in *N. crassa, C. heterostrophus* and *Magnaporthe grisea* showed that null mutants are sensitive to osmotic stress and resistant to the carboximide and phenylpyrrole classes of fungicides (Yoshimi *et al.*, 2004; Motoyama *et al.*, 2005). In *N. crassa* OS-1 has also been shown to be required for the production of asexual reproductive structures (most strikingly under hyperosmotic conditions). Moreover, heterologous expression of the *M. grisea* OS-1/HIK in *Saccharomyces cerevisae* yields a functional polypeptide with HK enzymatic activity and the ability to induce phosphorylation of HOG1 in response to the addition of phenylpyrroles, dicarboximides and aromatic hydrocarbon fungicides (Motoyama *et al.*, 2005).

The abuse in the use of dicarboximide fungicides to control the necrotrophic plant pathogen *Botrytis cinerea* has led to the occurrence of resistant strains and resistance correlates with changes in the locus Daf1 (Leroux *et al.*, 2002). Cui *et al.* (2002) determined that the Daf1 locus corresponds to BOS1 gene, which encodes the osmosensing histidine kinase *NIK1* orthologue. Furthermore, it was determined that single point mutations in BOS1 resulted in amino-acid changes that confer both, dicarboximide resistance and osmosensitivity. In a further study, Catlett *et al.* (2003) determined that BOS1 was the unique HK class III in *Botrytis cinerea*. When evaluated for virulence, bos1-null mutants were significantly less virulent than the wild type strain with smaller lesion sizes at 3 and 10 days post-inoculation of beans and tomato leaves (Viaud *et al.*, 2006). Complemented mutants showed similar size lesions than wild type strain, confirming that bos1 was responsible of the decrease in virulence.

In *Alternaria brassicicola*, another phytopathogenic fungus, mutants highly resistant to dicarboximides were isolated from laboratory (Yoshimi *et al.*, 2004) and the field (Iacomi-Vasilesc *et al.*, 2004). Although the cellular targets of phenylpyrroles and dicarboximides on these mutants were not identified, it was shown that both of them stimulate glycerol synthesis

in *N. crassa*. This observation and the fact that osmosensitive (os) mutants of *N. crassa* were also found to be resistant to dicarboximides and fludioxonil suggests that the response to osmotic stress and fungicide resistance might be linked (Fujimura *et al*., 2000). Further studies in this fungus showed that mutations in the 90 N-terminal amino acid repeats of the histidine kinase Nik1p/Os-1p are responsible for both, fungicide resistance and osmotic sensitivity (Dry *et al*., 2004).

Avenot *et al*. (2005) isolated from the field, *Alternaria brassicicola* resistant to iprodione and fludioxonil. They identified frameshift and nonsense mutations in the AbNIK1 histidine kinase gene. These strains were osmosensitive and virulent. These data suggest that histidine kinase class III play different roles during the infectious process of these phytopathogenic fungi. These fungi belong to different phyla; *B. cinerea* is a leotiomycete and *Alternaria brassicicola* is a dothideomycete and they differ in their infection processes and host range. *B. cinerea* is a necrotrophic and polyphageous pathogen of different plant organs including leaves and fruits, meanwhile *Alternaria brassicicola* is a seedborne necrotrophic pathogen of Brassicaceae. The differential functions of the class III HK in these pathosystems may be linked to different fungal strategies to handle host osmolarity during plant colonization.

The sensitivity of these strains to osmotic stress was evaluated by measuring their mycelial growth on PDA containing 4% NaCl. Among these mutants five fungicide resistant strains, denominated Abra43Fs, Abra20Fs, AbraRo10FS, Abra1662FS and AbraCMFS, displayed different level of osmotic sensitivity with only 62% inhibition for Abra43FS and 68-71% for the other isolates.

Using the primers designed by Alex *et al*. (1996) to amplify putative histidine kinases, a single 0.4 kb genomic DNA fragment was amplified from the *Alternaria brassicicola*. Using a PCR-walking procedure the 3´and 5´sequences of the histidine kinase gene were obtained. Southern blot of genomic DNA isolated from the fungicide sensitive Abra43FS and the fungicide resistant Abra40FR strains showed the AbNIK is a single copy-gene sequence. The predicted 3,987 pb ORF was interrupted by six introns ranging from 50 bp to 58 bp in size. The predicted 1329 amino-acid protein sequence exhibited all the characteristic features of histidine kinases, including the conserved H-, N-, D-, G1- and G2-boxes. The overall amino acid sequence identity of AbNIK1p with BmHK1p, BcOS1p and NcOS1p, three two-component osmosensing histidine kinases from *Cochliobolus heterostrophus*, *B. cinerea* and *N. crassa*, was 92, 64 and 60%, respectively. At the nucleotide level, AbNik1 has >80% identity with BmHK1 genes.

Recently, in the pathogenic basidiomycete *Criptococcus neoformans* a two-component-like multiple sensor kinase (Tco1) was isolated; it was related to diverse functions, including mating, stress response, sexual reproduction, melanin biosynthesis and virulence, by regulating the Psn2-Hog1 pathway. These findings suggested that the two-component system histidine kinase is related to fruiting body (mushroom) development (Bahn *et al*., 2006).

In another report, a developmentally regulated two-component histidine kinase Le.nik1 gene was described in the basidiomycete *Lentinula edodes*. The expression profiles during different developmental stages and under osmotic stress and the transcript localization during the development of fruiting bodies support that Le.nik1 may be related to osmoregulation of primordium formation and mushroom development.

The full-length cDNA of Le-nik1 is 6.29 kb long (GenBank No. DQ973528). The genomic sequence is 7.8 kb. (GenBank no. DQ973527). Primary transcript contain 22 introns of sizes ranging from 51 to 327 bp, in addition to 87 nucleotides upstream of the Le-nik start codon. Le.nik1 is predicted to encode a protein comprising 1874 amino acids with a molecular weight of 202.5 kDa and a pI of 5.78 (Bahn *et al*., 2006).

Similar to other NIK1 orthologues, Le.nik1 has a unique N-terminal region consisting of HAMP domain repeats. Closely following the HAMP repeats is the kinase core, composed of a dimerization domain and an ATP/ADP phosphotransfer domain, responsible for ATP binding and directing kinase transphosphorylation. Le-nik1 contains all five conserved amino acid motifs of a typical histidine kinase. Near to the C-terminus of Le-Nik1 lies the receiver domain (response regulator) where the Asp residue of the phosphorelay locates (Wolanin *et al*., 2002).

The expression of Le.nik1 is higher in the primordium than in the mycelium and it is up-regulated in the young and mature fruiting body compared with the primordium, indicating that Le.nik1 is not only related to primordium induction, but may also be involved with the development of fruiting bodies. When *Lentinula edodes* was grown under osmotic pressure (mannitol 1 M in culture medium) mycelial growth decreased when mannitol increase. Northern blot analysis revealed that Le.Nik1 transcript was highest in the medium supplemented with mannitol 1 M, compatible with the role of NIK1 in osmosensing and osmoregulation.

PHOSPHORELAYS AS POTENTIAL TARGETS AGAINST PATHOGENS

The His-Asp phosphorelay pathways have attracted attention as potential therapeutic targets, since it was thought for long time that they were absent in animals, particularly humans. However, currently there is little progress in developing of anti phosphorelay compounds that may be used as therapeutic agents in order to restore or improve human health (Chauhan and Calderone, 2008). In contrast, in pathogenic organisms (bacteria and fungi) that attack plants the indication of the His-Asp phosphorelay as a suitable target arose when it was demonstrated that phenylpyrroles and dicarboxiimide interfere with osmoregulation, particularly in fungi where they target the HOG pathway (Knauth and Reichenbach, 2000; Vetcher *et al*., 2007; Dongo *et al*., 2009). Interestingly, homologues of the group III of histidine kinase have been detected in all the pathogen fungal species analyzed, including the important crop pathogens *Botrytis cinerea* (Knauth and Reichenbach, 2000), *Alternaria brassicicola* and *Cochliobolus heterostrophus* (Dongo *et al*., 2009). The conservation of the protein NIK1 between species is very high and conservation occurs on the entire protein, including the novel N-terminal 90 residue repeats that might couple it to a membrane receptor. Presence restricted to specific species (such as fungi), make the members of this group suitable targets to develop drugs against the fungal histidine kinase III relay pathway, with the hope that new drugs have enough specificity to help us to fight the emerging fungal strains today (Gullino *et al*., 2000; Dongo *et al*., 2009). Furthermore, members of the histidine kinase group III (Nik1 orthologues) have been detected in plant pathogenic fungi as key regulators of virulence. Thus the development of specific inhibitors should be an interesting alternative to control losses induced by fungi in agriculture.

CONCLUSIONS AND PERSPECTIVES

The research on fungal histidine kinases is still in its infancy. However, the use of model fungi, mutants and availability of sequenced genomes from several non-pathogenic and pathogenic fungi is helping to identify elements that exerts molecular or biochemical regulation of histidine kinase, but particularly in this review we focused on elements of the class III histidine kinase because of their physiological importance in plant pathogenic fungi.

It is established that group III of histidine kinase genes are present in filamentous fungi such as *Aspergillus nidulans, Candida albicans, Magnaphorte grisea, Neurospora crassa, Cochliobolus heterostrophus* and *Botrytis cinerea*. Mutations in this protein is responsible for osmotic sensitivity

(Elad *et al*., 2004; Motoyama *et al*., 2005), relating the group III of histidine kinase with osmotic sensing in filamentous fungi. Histidine kinase class III acts as a positive regulator of the Hog1-type MAPK pathway (Yoshimi *et al*., 2005). The ability to osmoregulate is key for success of the phytopathogen in the host. Some phytopathogens, i.e., during *Botrytis cinerea* colonization of some host tissues, such as ripe fruits, the fungus has to cope with high internal osmolarity, which produces severe osmotic stresses (De Jong *et al*., 1997).

Interestingly it has been demonstrated that mutations in members of the group III of histidine kinase confers resistance in filamentous phytopathogenic fungi against the specific fungicides phenylpyrroles, dicarboximides and hydrocarbons (Ochiai *et al*., 2001; Leroux *et al*., 2002; Dongo *et al*., 2009). Although, the mechanism of action of these fungicides is not clearly established, there are two hypotheses to explain how resistance is acquired; (1) first, it is suspected that these fungicides should share a common mode of action that in wild type strains interferes with DNA synthesis and the progress of the cell cycle; in contrast, in mutant strains it is supposed that the common mechanism of action (the target) is interrupted and then the mutant strains are insensible to these compounds (Leroux *et al*., 2002; Dongo *et al*., 2009); (2) a second hypothesis comes from the observations that the dicarboximide menadione is toxic because it induces lipid peroxidation in cardiocytes and fungi (Tzeng *et al*., 1995; Choi *et al*., 1997). Resistance of bos1-null mutants to dicarboximides may be produced by differences in the degree of lipid peroxidation between the mutants and the wild type strains.

Histidine kinase pathways have been proposed as targets to design drugs against pathogenic mammalian fungi (Stephenson and Hoch, 2002; Chauhan and Calderone, 2008); such proposal is quite interesting. However, recent findings shows that in mammals two-component inhibitors also inhibit some structurally related mammalian protein kinases indicating that the use of these inhibitors may be a two-edged sword. In mitochondria there are enzymes like nucleoside diphosphate kinase (NDPK, also known as human Nm23s) and which may also act as a transcriptional regulator and histidine kinase (Warner *et al*., 1997; Postel, 2003). NDPK autophosphorylates on one histidine on the active site (using nucleoside triphosphate as a substrate) and this phosphoryl group could then be transferred to histidine residues in other proteins. NDPK from rat liver could phosphorylate an histidine residue on ATP-citrate lyase (Warner and Vu, 2000). This evidence shows that NDPK could act as histidine kinase in animal cells (Warner and Vu, 2000).

α-ketoacid dehydrogenase kinase (BCKDHK) and pyruvate dehydrogenase kinase (PDHK) contain prototypical histidine kinase motifs (Tuganova *et al*., 2001; Davie *et al*., 1995), both enzymes auto-phosphorylate on a serine residue *in vitro* (Thelen *et al*., 2000). However, the possibility that BCKDHK and PDHK have also intrinsic histidine kinase activity has generated reasonable doubts regarding their absence in animal cells, particularly mammals (Lasker *et al*., 2002). In the case of PDHK the His-239 plays an essential role in the phosphotransfer reaction; mutation (sustitution) in the His-239 for Ala resulted in a 90% decrease in activity. However, to date, there is no *in vivo* evidence of BCKDHK being involved in a signaling event similar to that which is well-established for the two component histidine kinases (Embley *et al*., 2003).

Additional evidence supporting the presence of HK-like systems in mammalian cells comes from the use of the HK inhibitors. Eukaryotic elongation factor-2 kinase (eEF-2 kinase) is involved protein synthesis. The eEF-2 kinase has no known histidine kinase activity but it is inhibited by 2-methylimidazolium iodide (NH-125) and structurally related compounds, which are well known histidine kinase inhibitors (Arora *et al*., 2003, 2004). Inhibition of eEF-2 kinase and BCKDHK by

anti-microbial inhibitors targeting histidine kinases provides a timely cautionary note to use these drugs in mammals (Gullino *et al*., 2000), even when two component histidine kinases are absent in humans and other mammals, this not preclude the inhibition of proteins like BCKDHK, PDHK or other proteins that may be structural analogues of the fungal or bacterial target proteins.

Grouping together all the findings regarding histidine kinase from class III, they support the idea that this particular class of histidine kinases enclose the potential to be used as the particular targets to develop specific inhibitors of pathogenic fungi for both, those that affect humans, such is the case of *Candida albicans* or those that causes important losses in the agriculture, such is the case of *Alternaria brassicicola* and *Botrytis cinerea*. The former is an opportunistic pathogen that attacks humans with severely immuno-compromised system (Calera and Calderone, 1999; Yamada-Okabe *et al*., 1999) and the latter's affect plants that are important sources of human and animal food. The findings that orthologs of the NIK1 are present in both kinds of pathogens supports such suggestion. In accordance with this idea, future research must be focused to find natural drugs or in the synthetic design of specific drugs whose target must be the class III histidine kinase. The development of such compound should alleviate the actual challenge represented by human and plant fungal pathogens.

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