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INDEX

Abstract

Multicellular organisms must be able to survive to mechanical damage resulting from predation or adaptation to hostile environments. *Trichoderma atroviride* responds to mechanical damage by activating cascades of regenerative processes and asexual reproduction (conidiation). Our evolving understanding of mechanisms in response to injury has been facilitated by genetic and transcriptional analyses, and physiological and biochemical characterization. During response to injury, key elements such as Ca^{2+} influxes, signal molecules such as Damage Associated Molecular Patterns (DAMPs), reactive oxygen species (ROS), mitogen-activated protein kinases (MAPKs) activation and calcium signaling establish a signal transduction process that triggers the injury response.

To understand the underlying early signaling events involved in the injury response, we evaluated molecules such as extracellular ATP (eATP) and Ca^{2+} on conidiation and MAPK phosphorylation. Indeed, application of exogenous ATP triggers conidiation and mimics the injury response by induction of specific genes including Lipoxygenase, phospholipase A2, catalases and components of calcium signaling. Voltage-gated calcium channels and intracellular pools promote elevation of $[Ca^{2+}]_c$ after injury. Mutants in the MAPK-encoding genes *tmk1* and *tmk3* were affected in wound-induced regeneration and conidiation, respectively. The MAPK Tmk1 pathway and calcium signaling appear to regulate genes involved in hyphal healing and regeneration, while Tmk3 is activated by Nox1-dependent ROS and promotes expression of conidiation genes, mainly. We conclude that in this fungus, eATP acts as DAMP. Our data indicate the existence of an eATP receptor in fungi; eATP triggers pathways that converge to regulate asexual reproduction genes that are required for injury-induced conidiation. In contrast, Ca^{2+} is more likely to act as a downstream second messenger. Finally, the early steps of the mechanical damage response in *T. atroviride* share conserved elements with those known from plants and animals.

Keywords: Injury response, regeneration, eATP, DAMPs, cytosolic calcium, ROS, MAPK.

Resumen

La respuesta al daño mecánico es crucial para la supervivencia de cualquier organismo multicelular, ya que permite su adaptación en ambientes hostiles. *Trichoderma atroviride*, responde al daño mecánico mediante la activación de vías que conducen a la regeneración y reproducción asexual. Para comprender los mecanismos involucrados en la respuesta a daño, hemos hecho análisis genéticos, caracterización fisiológica y bioquímica y finalmente un análisis transcripcional. Durante esta respuesta, elementos clave tales como flujos de Ca^{2+}, moléculas señal como los DAMPs por sus siglas en inglés: Damage Associated Molecular Patterns, especies de oxígeno reactivo (ROS), activación de proteína cinasas activadas por mitógenos (MAPKs) y activación de la vía de calcio, establecen una interacción para desencadenar la respuesta a herida. Se evaluaron moléculas como ATP extracelular (eATP) y Ca^{2+} sobre la conidiación y la fosforilación de MAPKs. La aplicación exógena de ATP desencadena la conidiación y mimetiza la respuesta a herida mediante la inducción de genes específicos como la lipoxigenasa, fosfolipasa A2, catalasas y componentes de la señalización del calcio. Los canales de calcio controlados por voltaje y los depósitos intracelulares de este ion promueven la elevación de $[Ca^{2+}]_c$ después del daño mecánico. Mutantes en dos MAPKs Tmk1 y Tmk3 fueron afectadas en la regeneración y conidiación después de la herida, respectivamente. La MAPK Tmk1 y la vía calcio regulan genes relacionados al proceso de saneamiento y regeneración de hifas, mientras que Tmk3 es activada por ROS de manera dependiente de Nox1 y participa principalmente en la conidiación. Concluimos que, en este hongo, eATP actúa como DAMP. Nuestros datos indican la existencia de un receptor eATP en hongos. El eATP desencadena vías que convergen para regular genes involucrados en la reproducción asexual que se requieren para la conidiación inducida por lesiones. Por el contrario, el Ca^{2+} es más probable que actúe como un segundo mensajero. Finalmente, los procesos tempranos en la respuesta al daño en hongos filamentosos incluyen mecanismos que son conservados entre plantas y animales.

Palabras clave: Respuesta a daño, regeneración, eATP, DAMPs, calcio citosólico, ROS, MAPK.

1. Introduction

The wound response and regeneration processes have typically been studied in model organisms, due to their regenerative ability. Although, the molecular mechanisms involved in these processes are still poorly understood, recent studies reveal multiple similarities in the wound response of mammals (Crisan et al., 2016), plants (Duran-Flores and Heil, 2014; Heil et al., 2012; Martinez-Medina et al., 2016), insects (Krautz et al., 2014) and fungi. These similarities include the basic chemical structure of signal molecules, pathways involved in their perception and transduction, and the subsequent transcriptional response. Wound healing and regeneration of lost structures can take weeks in some vertebrates such as salamanders or axolots (Currie et al., 2016; Tweedell, 2010) and more quickly (days) on tissue specific regeneration in mammals (rats or humans) (Taub, 2004). Some animals with high regenerative capacity develop a mass of cells capable of growth and regeneration into organs or body parts known as blastema (Tanaka and Reddien, 2011). Morphological processes are observed after wound and classified according to species. The long regeneration time of limb/tissue, for example in axolotls (**Fig. 1**), the handling complexity and specific stages of development of regenerative animal models mentioned, represent certain disadvantage. In contrast, fungi are excellent models to study signal transduction pathways, since they often have equivalent pathways to those of other multicellular eukaryotic organisms and very short regeneration time (Hernandez-Oñate et al., 2012). Fungi like other organisms have natural predators including fungivorous nematodes and arthropods (Caballero Ortiz et al., 2013); consequently fungi must have effective mechanisms to contend with and survive to mycelial injury. *Trichoderma atroviride* is a fungus belonging to the ascomycetes, which are common soil inhabitants, establishing symbiosis with plants and parasitizing phytophatogenic fungi (Chet et al., 1981; Marra et al., 2006; Viterbo et al., 2005). This fungus regenerates quickly upon mycelial injury; a new hypha appears within the first hour after damage; moreover, it develops mature asexual structures 24h after injury (Hernandez-Onate et al., 2012).

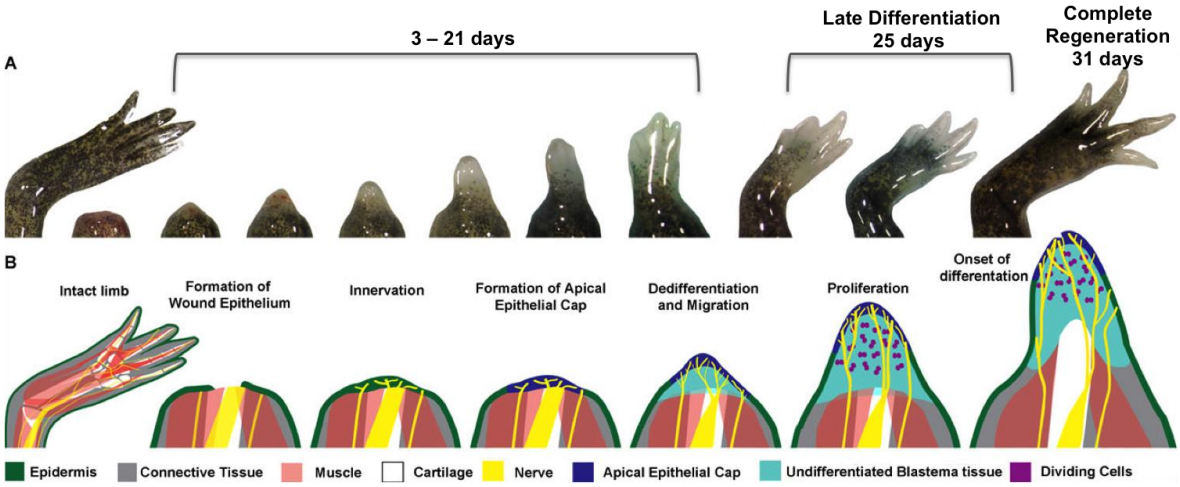


Figure 1. Axolotl limb regeneration. **A**. Live images of time course of limb regeneration: 1 day, 7, 9, 11, 13, 15, 17, 21, 25, and 31 days post amputation (consecutively to the right). **B**. The key steps in the regenerative process are highlighted during blastema development. Taken and modified from (McCusker et al., 2015).

1.1 Regeneration and asexual reproduction after injury in fungi

The concept of regeneration is not commonly used in fungi, possibly because it is a largely unexplored area. Nevertheless, most filamentous fungi can reinitiate hyphal growth from the cell adjacent to a cell that has been mechanically damaged. Albeit only in certain fungal cell differentiation and asexual/sexual development after injury has been observed; therefore, the whole process of reinitiation hyphal growth after injury, including the participation of conserved mechanisms and cell differentiation, can be considered regenerative processes in fungi.

Hyphal compartments from the ascomycetes and basidiomycetes are interconnected by septal pores. These structures allow a constant flow of protoplasm across the cells (Cole, 1986). In ascomycetes, when hyphae are damaged or hyphal lysis occurs, peroxisome-derived vesicles called Woronin bodies prevent excessive cytoplasmic loss and seal the septal pore. In basidiomycetes the septal pore cap (parenthosome) plays the role of the Woronin body and localizes at the septal pore in *Schyzophyllum commune* and *Trichosporon sporotrichoides* (Muller et al., 1998).

In *Neurospora crassa* and *Aspergillus nidulans*, upon hyphal damage, a single Woronin body is typically translocated to the septal pore nearest to the point of injury and new hyphal tips are generated from this position. The sealing of the pore reduces loss of cytoplasmic content to prevent cell death (Trinci and Collinge, 1974), resulting in reinitiation of growth and hyphal reconnection in both fungi (Jedd, 2011). Recently, a group of septal pore-associated (SPA) proteins controlling diverse aspects of septal organization associated with Woronin bodies and that are involved in cell wounding in *N. crassa* were identified. One of these proteins, SPA14, contains an annexin domain and is likely calcium regulated (Lai et al., 2012). Nevertheless, the cumulative knowledge on the mechanisms involved in the injury response and regeneration in filamentous fungi is still rather limited. As mentioned before, some fungi initiate an asexual or sexual reproduction program after damage. The spores or conidia produced in response to damage can then be dispersed by the wind or hitching a ride on an animal. The most common mode of asexual reproduction is through the formation of asexual spores also known as conidia. Conidiophores are specialized structures that produce conidia (a type of asexual spores) that are released to the environment. Interestingly, only a few fungi have been shown to develop asexual/sexual structures after mechanical damage: *Schizophyllum commune* (Leonard and Dick, 1968), *Sclerotium rolfsii* (Henis et al., 1965), and *Aspergillus flavus (Hernandez-Onate and Herrera-Estrella, 2015)* produce such structures in response to mycelial wounding. However, Hernández-Oñate and coworkers (2012, 2015) showed that several species of *Trichoderma* such as *T. harzianum, T. hamatum, T. virens* and *T. atroviride* also conidiate in response to injury (**Fig. 2A-B**).

T. atroviride responds to mechanical damage with a morphogenetic change initiated by the transcriptional activation of regenerative processes. One hour after injury, growth of the new hypha is evident and 24 hours later the formation of asexual reproduction structures around the damaged area is observed (**Fig. 2A)**. Moreover, NADPH oxidase (Nox1) dependent ROS production is essential for this developmental response to damage (Hernandez-Onate et al., 2012). Recently, Medina-Castellanos (2014) and coworkers showed that MAPK pathways are activated in response to wounding and addition of extracellular ATP. Consequently, it has been proposed that this molecule could be an “alarm signal” released from broken hyphae, known as Damage Associated Molecular Patterns (DAMPs) in plants and animals.

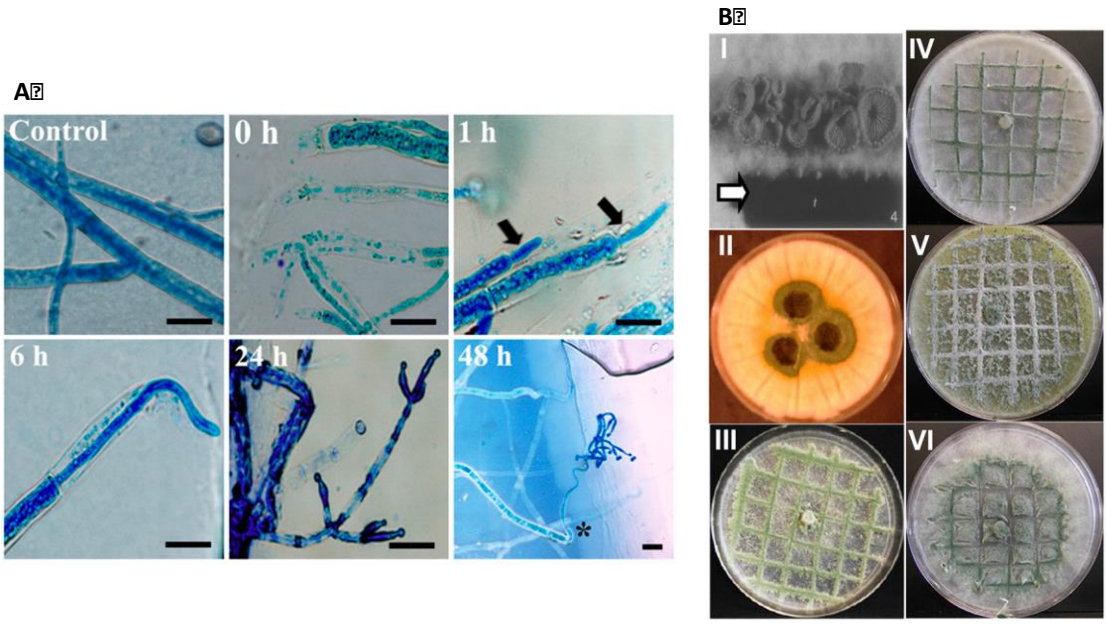


Figure 2. Formation of reproductive structures in response to injury. A. Conidiophores are formed from regenerating hyphae. An asterisk indicates the generation of conidiophores from the regenerated hyphae. Scale bar = 20 μm. **B.** I *S. commune* fruiting body formation triggered by release of fruiting-inducing substances after hyphal lysis; arrow shows the contact zone with FIS (Leonard and Dick 1968). II Conidiation induced by mechanical damage in *A. flavus* (photograph kindly provided by Dr. Anna Calvo). III–VI Photograph showing the conidiation in the injury area in III *T. atroviride* (Hernández-Oñate et al. 2012), IV *T. harzianum*, V *T. hamatum*, and VI *T. virens.* Taken and modified from Hernández-Oñate et al. 2012; 2015.

1.2 Damage associated molecular patterns and their perception

In other systems, reliable signals of tissue disruption are known to comprise fragments of the extracellular matrix, extracellular molecules such as ATP, adenosine, RNA or DNA, and certain proteins or protein fragments (Chen and Nuñez, 2010; Zeiser et al., 2011; Heil, 2012). These warning signals are known as DAMPs (Bianchi, 2007; Heil and Land, 2014).

In plants, several DAMPs have been identified; the best-characterized class are polypeptides/peptides produced from larger precursor proteins. The role of one of these DAMPs, systemin, during wound response in tomato has been studied in depth. This molecule activates the jasmonic acid (JA) pathway and the systemic response to prevent future infections (Narvaez-Vasquez and Ryan, 2004). Similarly, plant elicitor peptides that activate defense gene expression are classified as DAMPs. Other DAMPs are those found

in the extracellular matrix, called oligogalacturonides (OGs), derived from the pectic polysaccharide homogalacturonan, and extracellular adenosine 5'-triphosphate ATP (eATP). Both molecules can be released mechanically or by hydrolytic enzymes produced by pathogens. This class of molecules induces innate immune response, MAPK3/MAPK6 activation, callose deposition, ROS production and Ca^{2+} influxes (Choi and Klessig, 2016). Recently Choi and coworkers (2014) discovered a specific receptor for extracellular ATP, through the mutant *dorn1* (does not respond to nucleotides). They also showed that the addition of eATP induces innate immune responses, such as increases of cytosolic Ca^{2+}, Mitogen Activated Protein Kinases (MAPK) pathways activation (MAPK3/MAPK6) and induction of genes involved in the biosynthesis of JA and ethylene (Choi and Klessig, 2016). Some DAMP receptors have been identified, such as those of the leucine-rich repeat receptor kinase (LRR RK) class including: PEPR1, PEPR2, FLS2 (flagelin receptor), LecRK-I.9 (ATP receptor), BRI1-Associated Kinase (BAK1), BAK1-Like kinase 1 (BKK1) and wall-associated kinase (WAK1). High Mobility Group Box (HMGB) proteins, first identified in mammals represent a fourth class of DAMPs. In *Arabidopsis thaliana* 15 genes encoding HMGB-box domain – containing proteins have recently been reported as novel DAMPs in plants. For instance, the *At*HMGB3 protein, is released into the apoplast during the interaction with the necrotrophic fungus *Botrytis cinerea*, activating defense pathways related with JA/ethylene biosynthesis (Choi et al., 2016).

Around 26 DAMPs have been identified in animals, including purines, pyrimidines, DNA (unmethylated CpG), low-density lipoproteins, N-formyl peptides and a variety of proteins. One of the first characterized DAMPs in animals is the High Mobility Group Box 1 (HMGB1) protein, which is among the most important chromatin proteins. It interacts with nucleosomes, transcriptional factors and histones (Bianchi and Agresti, 2005), and also functions as DAMP, since it is released from damaged or severely stressed cells and induces cytokines and pro-inflammatory factors, that are often recognized by Toll Like Receptors (TLRs) (Choi and Klessig, 2016).

For decades, it was considered that the function of ATP was exclusively that of a neurotransmitter, involved in muscle contraction and, cell death and inflammation, in addition to being a central molecule of energy in all-living organisms. Although recently, a possible role

of ATP as a signal molecule and DAMP has been explored both in animals and plants. This molecule has been studied in cell to cell communication, but recently it has been established

that large increases in [eATP] are associated with cell death, and serve as a key “danger” signal in inflammatory processes in zebra fish (de Oliveira et al., 2014), humans (de Oliveira et al., 2014; Trautmann, 2009), algae (Torres et al., 2008) and wound response in plants (Tanaka et al., 2014). Typically, two types of receptors are involved in perceiving eATP or nucleotides; receptors coupled to heterotrimeric guanine nucleotide binding proteins (GPCRs) such as P2Y receptors and ligand-gated ion channel P2X receptors. These receptors trigger the increase of intracellular calcium, MAPKs activation and accumulation of Reactive Oxygen Species (ROS) (Trautmann, 2009). Animals and plants share similar signaling components, however, DAMP receptors exhibit an extensive variety of structures and domains, to sense the same molecule or danger signal in different organisms.

1.3 MAPKs activation and ROS signaling in wound response and regeneration

Several signaling pathways have been shown to play a role in outgrowth after injury. Mitogen Activated Protein Kinases are responsible for the signal transduction of many stimuli or stresses by consecutive phosphorylation until the third MAPK. Protein kinases, comprising 2% of eukaryotic genomes, remove phosphate from ATP and covalently attach it to the free hydroxyl groups of serine, threonine, or tyrosine residues. The intracellular signaling after injury perception must be activated quickly and MAPK cascades have been shown to play a key role in transduction extracellular signals to cellular responses.

In plants, at least two MAPKs, salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), are rapidly activated by wounding or insects (Hettenhausen et al., 2015). In *A. thaliana* MPK6 and MPK3 are phosphorylated after wounding (Ichimura et al., 2000). The MAPKs activation is reported in regeneration as well.

The PI3K and ERK pathways are essential for axon regeneration after spinal cord injury (Atwal et al., 2000; van Niekerk et al., 2016). Moreover, ERK signaling promotes acetylation of regeneration-associated promoters (Puttagunta et al., 2014). The stress-activated C-Jun–

NH_2–kinase (JNK)-dependent apoptotic cell death is crucial to coordinate tissue renewal and remodeling required to regenerate the planaria body (Almuedo-Castillo et al., 2014). Recently epigenetic reprogramming during tissue regeneration was reported, since JmjD3, a demethylase involved in activating blastema cells is regulated by MAPK/ERK signaling in zebra fish (Katsuyama and Paro, 2011; Tan et al., 2015). Moreover, MAPK/CREB pathway triggers apoptosis-induced compensatory proliferation in hydra head regeneration (Chera et al., 2011).

In most filamentous fungi, there are three MAPKs pathways that belong to the so-called filamentous growth, cell wall integrity and osmotic stress response pathways. In *T. atroviride* the MAPKs are named Tmk1, Tmk2 and Tmk3, and their corresponding orthologues in yeast are Kss1/Fus3, Slt2 and Hog1 (Mendoza-Mendoza et al., 2003; Delgado-Jarana et al., 2006; Reithner et al., 2007; Zeilinger and Omann, 2007). These MAPKs are involved in several biological process, such as asexual and sexual reproduction, general stress response, spore germination, cell fusion, control of secondary metabolism and mycoparasitism (Mendoza-Mendoza et al., 2003; Delgado-Jarana et al., 2006; Reithner et al., 2007; Fleissner et al., 2009; Kumar et al., 2010; Lara-Rojas et al., 2011; Lichius et al., 2012; Bayram et al., 2012). Defective sexual and asexual development resulting from MAP kinase mutations has been reported in *Magnaporthe grisea* (Xu et al., 1998), *Fusarium graminearum* (Hou et al., 2002), *Neurospora crassa* (Lichius et al., 2012) and *Aspergillus nidulans* (Wei et al., 2003). Furthermore, It was recently reported that in *T. atroviride* the MAPK Tmk3 is rapidly phosphorylated upon light exposure and that it regulates the expression of light responsive genes and cellular stress in general (Esquivel-Naranjo et al., 2016). Interestingly, MAPKs are not only known to be activated by perception of ligand but are also activated by ROS (Jalmi and Sinha, 2015).

The ROS with physiological significance include the superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$), which are produced by living organisms as a result of normal cellular metabolism. These species have different chemical properties and specific biological targets. The maintenance of redox homeostasis is key for avoiding oxidative stress damage of lipids, proteins, carbohydrates and nucleic acids (Birben et al.,

2012). Recently, it has been shown that ROS participate as signaling molecules, in inflammation and tissue injury (Mittal et al., 2014). ROS signaling in plants is essential for the wound response to prevent future infections and healing the wound. Moreover, ROS induce repair mechanisms in the damage zone, DNA repair and programmed cell death (Heil et al. 2012; Mittler et al. 2011; (Duran-Flores and Heil, 2014).

Plant NADPH oxidases are involved in host defense via the hypersensitive response, and in development via regulation of plant cell expansion through the activation of Ca^{2+} channels (Foreman et al., 2003). In zebra fish, H_2O_2 derived from superoxide produced by the NADPH oxidase (Duox1) is responsible for the formation of a ROS gradient in tissue injury and triggers the inflammatory signaling (de Oliveira et al., 2014).

In most fungi there are three subfamilies of NADPH oxidases; NoxA, NoxB and NoxC, which have been implicated in asexual or sexual reproduction, pathogenesis, ascospore germination and plant–fungal interactions (Takemoto et al., 2007). A study on an NADPH oxidase of *T. harzianum* by Montro-Barrientos *et al.* (2011) revealed that the nox1 gene was implicated in the production of ROS and in the secretion of hydrolytic enzymes. In this sense, a transcriptome analysis of the response to injury of *T. atroviride* revealed a cluster of genes involved in oxidative stress including ROS scavengers, such as catalases, superoxide dismutases and thioredoxines. Moreover, the NADPH-oxidase complex (Nox1) and the regulatory subunit (NoxR) are essential for wound-induced ROS production at the hyphal tip as well as in the production of conidia (Hernandez-Onate et al., 2012). Interestingly, in plants, oxidized lipids such as jasmonic acid (JA), known as oxylipins are formed enzymatically, during wounding and pathogen infection (Lopez-Raez et al., 2010). Oxylipins biosynthesis is reported in fungi as well (Fischer and Keller, 2016; Lopez-Raez et al., 2010). Hernández-Oñate (2012) and coworkers identified a set of genes encoding components of oxylipin production, particularly a lipoxygenase, induced at early stages of the response to injury. This enzyme determines programed spore germination in *A. fumigatus* (Fischer et al., 2017).

1.4 Calcium signaling

All organisms employ simple molecules to sense the environment. Ca^{2+} ions are considered second messengers that activate signal transduction pathways through enzyme targets, such as the calcium sensor protein calmodulin, calcium-dependent protein kinases (CDPKs), and EF-hand motif proteins (Arimura and Maffei, 2010). The

basal concentration of Ca^{2+} in the cytoplasm is around 100 nM, 20,000 to 100,000 fold lower than typical extracellular concentration (Clapham, 2007). To maintain this low concentration, Ca^{2+} is actively pumped from the cytosol to the extracellular space, the endoplasmic reticulum (ER), and sometimes into the mitochondria. Certain proteins of the cytoplasm and organelles act as buffers by binding Ca^{2+}. Signaling occurs when the cell is stimulated to release calcium ions (Ca^{2+}) from intracellular stores, and/or when calcium enters the cell through plasma membrane ion channels (Clapham, 2007).

In fungi $[Ca^{2+}]_{cyt}$ increases often appear as transient spiking or repetitive oscillations, where the amplitude, frequency, shape, and duration are determined by the specific stimulus. Oxidative stress, osmotic stress and mechanical perturbation provoke distinct waves of Ca^{2+} in *A. fumigatus* (Munoz et al., 2015) and in plant cells (Cheng et al., 2015). In agreement with these reports, in *T. atroviride* the transcription of a set of genes involved in calcium signaling, including Ca^{2+} calmodulin-dependent kinase-1 (CAMK-1), the calcineurin-responsive zinc finger transcription factor (CRZ1) and transporters of calcium is induced (Hernandez-Onate et al., 2012). Furthermore, it has been suggested that Ca^{2+} ions regulate the recruitment of vesicles at the injury sites in mammal cells. Three distinct mechanisms of plasma membrane lesion repair have been described: formation of “lipid-patches", in which intracellular vesicles fuse with one another to form membrane patches; “lysosomal exocytosis”, where an acid sphingomyelinase (aSMase) is secreted to the extracellular space through lysosome exocytosis and, “macro-vesicle shedding”, a process that involves the assembly of endosomal sorting complex required for transport (ESCRT) machinery (Cheng et al., 2015) (**Fig. 3**). Indeed, recent studies suggest that not only extracellular Ca^{2+} but also the release from intracellular pools may also be important for plasma membrane resealing (Cheng et al., 2015). Interestingly, in plants it has been reported that a glutamate

receptor like protein, a family of calcium-permeable ion-channel proteins, such as channels involved in eliciting electrical signals during hervibory or wounding (Mousavi et al., 2013).

The synergic participation of signaling pathways represents an evolutionary-conserved mechanism to launch a regenerative process. Accordingly, in this work the key mechanisms or factors involved in hyphal regeneration were analyzed. Therefore, the early steps of the response to mechanical damage were studied and identified a set of “regeneration genes” in *T. atroviride.* To the best of our knowledge, the present study is the first that contributes to understanding the signaling pathways involved in the wound response in filamentous fungi.

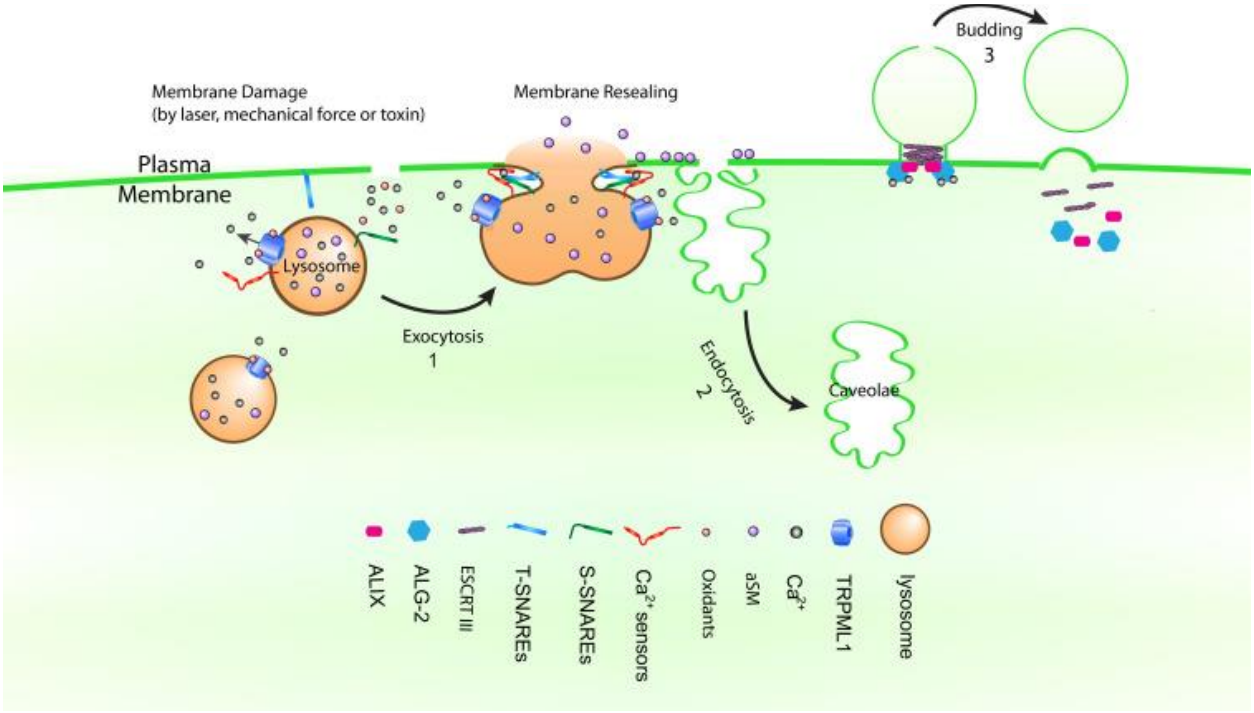


Figure 3. Three working models for membrane repair. In the “lipid-patch” model (1), TRPML1, Syt-VII, dysferlin, and SNAREs participate in membrane repair. Upon the incursion of membrane damage, an influx of oxidants and Ca^{2+} promotes TRPML1 conducted lysosomal Ca^{2+} release, activating Syt-VII and other Ca^{2+} sensors. Subsequently, lysosomal exocytosis is triggered to reseal the disrupted membranes. In the “endocytic removal” model (2), lysosomal exocytosis mediates the release of aSMase to catalyze ceramide-dependent rapid lesion removal by caveolar endocytosis. In the “macro vesicle shedding” model (3), an injury-triggered Ca^{2+} surge recruits ALG-2 to the injury site. Taken from (Cheng et al., 2015).

2. Hypothesis and Objectives

Hypothesis

In *Trichoderma atroviride* injury is perceived through DAMPs and the response to injury is regulated by MAPKs and Ca^{2+} signaling pathways.

General Objective

To identify the signaling molecules that trigger the response to damage and to determine the role of the MAPKs Tmk1 and Tmk3 in injury-induced conidiation and regeneration in *T. atroviride*.

Specific Objectives

1. Determine the role of the MAPKs Tmk1 and Tmk3 in the response to injury.
2. Determine if the activation of Tmk1 and Tmk3 depends on ROS production generated by the NADPH oxidase 1 (Nox1).
3. Identify putative signaling molecules involved in the response to injury.
4. Identify clusters of genes, whose expression co-relates with regeneration and injury induced conidiation.

3. Materials and methods

3.1 Strains and Culture Conditions

T. atroviride IMI 206040 was used as wild type (WT) strain. The *T. atroviride Δtmk1, Δtmk3, Δnox*1, *Δnox*2, and *Δnox*R mutant strains were previously generated in Dr. Herrera-Estrella laboratory by Edgardo Ulises Esquivel-Naranjo and have been described earlier (Hernandez-Onate et al., 2012; Medina-Castellanos et al., 2014). All strains were propagated on potato dextrose agar (PDA). The *A. fumigatus* strain carrying the calcium reporter GCamP6 (Akerboom et al., 2012) was generated by transformation with plasmid pRSK379 (Wagener et al., 2008) and kindly provided by the laboratory of Dr. Nick Read (University of Manchester) and cultured at 25°C or 37°C in Minimal Vogel´s Medium (MMV). To visualize actin and intracellular calcium dynamics during growth and injury it were generated *T. atroviride* strains with plasmids pEM12 and pEM13, which carry a calcium sensor GCamP6 (pEM12) and a LifeAct-GFP protein fusion (pEM13), under the control of the *T. reesei* pyruvate kinase (*pki*) promoter.

3.2 Plasmid Construction

All plasmids used and generated are described in **Table 1.** Ampicillin and chloramphenicol were used for selection and maintenance in bacterial cells. Oligonucleotides used to generate constructs and validate them are indicated in **Table 2.**

To generate pEM12, the sequence of GCamP6 (Calmodulin::GFP) was obtained from plasmid pSK379 (Wagener et al., 2008) and amplified by PCR using the primers GCaMP6-EcoRI-FW and GCaMP6-SalI-RV, which added the restriction sites *Sal*I and *Eco*RI that were used to clone the PCR product into plasmid pUE10. pUE10 carries the *T. reseei* pyruvate kinase promoter, the *T. atroviride* blu17 terminator, and a hygromycin resistance cassette. The construct *Trpki*^{P}*::*GCamP6*::Tablu17*^{t} contained in pEM12 was verified by PCR with oligonucleotides GCaMP6-FW and GCaMP6-RV (**Supplementary Figure 1s**).

To generate pEM13, the sequence of LifeAct-EGFP (Belin et al., 2014) was obtained from plasmid pRS472 (kindly provided by Dr. Rosa Mouriño-Pérez) and amplified by PCR using the primers: LifeAct-XbaIpUE10-FW and LifeAct-EcoRIpUE10-RV, which added the restriction sites *Xba*I and *Eco*RI. The PCR product was then cloned into the *Xba*I and *Eco*RI sites of plasmid pUE10. The resulting construct *Trpki*^{P}*::*LifeAct-GFP*::Tablu 17*^{t} contained in pEM13 was verified by PCR with oligonucleotides Ppki-FW and TBlu17-RV (**Supplementary Figure 2s**).

Table 1. Plasmids used and generated in this study.

Name	Purpose	Resistance for selection in fungi	Target locus	Reference
pUE10	Cloning vehicle for targeted insertion of GCamP6 under Pyruvate kinase promoter [*Trpki*^{P}] and *Tablu17* terminator [*Tablu 17*^{t}]	hygromycin [*hph*]	N/A	(Balcazar-Lopez et al., 2016)
pSK379	Vector containing calcium reporter GCamP6 for cloning in pUE10	pyrithiamine [*ptrA*]	ORF GCamP6, between [P*gpdA*] and [*his2A*^{t}]	(Wagener et al., 2008)
pEM12	Integrative vector for targeted insertion of GCamP6 into *T. atroviride* genome via *Blu17*^{t}	hygromycin [*hph*]	*Trpki*^{P}*::*GCamP6 *::Tablu 17*^{t}	Derived from pUE10, this study
pEM13	Integrative vector for targeted insertion of LifeAct-GFP into *T. atroviride* genome via *Blu17*^{t}	ygromycin [*hph*]	*Trpki*^{P}*::LifeAct-GFP::Tablu 17*^{t}	erived from pUE10, this study
TOPO® Cloning	Cloning vector for GCamP6	N/A	N/A	

Table 2. Oligonucleotides used in this study**.**

Name	Sequence	Use
GCaMP6-EcoRI-FW	CGG GCT GCA GGA ATTCATG GGT TCT CAT CAT CAT CAT CAT CAT G	Amplify the ORF GCaMP6
GCaMP6-SalI-RV	GGC CCT CGA GGT CGA CTCA CTT CGC TGT CAT CAT TTG TAC AAA C	Amplify the ORF GCaMP6, probe amplification for Southern blotting of pEM12
Ppki-FW	CGC CTT CCC GCA GCT CAG G	Probe amplification for Southern blotting of pEM12
TBlu17-RV	GGG GCA AAG ACA ATT GAT AGA ACA GCA	Diagnostic by PCR of pEM12
GCaMP6-FW	ATG GGT TCT CAT CAT CAT CAT CAT CAT G	Diagnostic by PCR of pEM12
GCaMP6-RV	TCA CTT CGC TGT CAT CAT TTG TAC AAA C	Diagnostic by PCR of pEM12
LifeAct-XbaIpUE10-FW	TGGCGGCCGCTCTAGAATG GGT GTC GCA GAT TTG ATC AAG	Amplify the LifeAct-GFP
LifeAct-EcoRIpUE10-RV	GCTTGATATCGAATTCTTACTTGTACAGCTCGTCCA TGCC	Amplify the LifeAct-GFP from

3.3 Transformation of *T. atroviride*

Plasmids pEM12 and pEM13 were used for PEG-mediated protoplast transformation of the wild type strain of *T. atroviride*. Briefly, $1x10^8$ conidia from *T. atroviride* stock cultures were inoculated in GYEC (Glucose Yeast Extract Casein hydrolized) liquid medium, and allowed to grow for 16-20 h with shaking (150–250 rpm) at 26–28°C. To obtain protoplasts, mycelium was filtered (0.2-0.5 g of mycelia) and then treated with lysing enzymes from Trichoderma harzianum (Sigma®) 60 mg dissolved in 6 mL of osmotic solution (50 Mm $CaCl_2$, 0.5 M manitol, 59 mM MES, pH 5.5 adjusted with KOH) and gentle shaking (100 rpm) for 2-3 h. Next, the protoplasts were filtered from cell wall debris by microfilters miracloth (100 and 50 µm size pore) as previously described (Turgeon et al., 1987). The optimal concentration of protoplast is 10^7-10^8. We used protoplasts and 10-20 µg of DNA or plasmid resuspended in osmotic solution, incubated on ice 20 min and then added polyethylene glycol 8000 (42ºC). The reaction was kept for 30 min at room temperature. Transformants cells were selected on plates containing 100 □g/ml hygromycin B (Fluka,

Sigma–Aldrich, Steinheim, Germany). After three rounds of single spore isolation, fungal DNA was isolated from transformant mycelia using standard protocols. Purified transformants were then verified by PCR and Southern blot.

3.4 Southern blot analysis

The *Δtmk1, Δtmk3, Δnox1, Δnox2*, *ΔnoxR* mutants and *T. atroviride* carrying pEM12 were verified by Southern blot, following standard procedures (Sambrook and Russell, 2001). Genomic DNA was digested with restriction enzymes, separated by electrophoresis in a 1% agarose gel, and transferred onto Hybond-N^+ membranes (Amersham). DNA fragments used as probes were labelled with [^{32}P] dCTP by random priming, using the Readyprime kit (Amersham) according to the manufacturer's specifications.

Genomic DNA of *Δnox1, Δnox2*, and *ΔnoxR* was extracted and digested with *Pst*I, *Sal*I and *Sph*I, respectively. The probes used to verify the *Δnox1, Δnox2 and ΔnoxR* mutants were a 2.76 kb, a 3.11 kb and a 3.17 kb fragment, respectively, containing the complete ORF and 1.5 kb at the 3´UTR in all the cases as shown in **supplementary figure 3s.** Genomic DNA of the *Δtmk1* and *Δtmk3* mutant strains was extracted and digested with *Pvu*II and *Eco*RI, respectively. The probes used to verify the *Δtmk1* and *Δtmk3* mutants (a 2.9 and a 3.3 kb fragments) included the complete ORF and 1.5 kb at the 5' UTR as shown in **supplementary figure 4s.** Genomic DNA of four transformants carrying pEM12 were obtained and digested with *Kpn*I; the length of the probe was 1.082 bp and included the GCamP6 cassette and the *Trpki* promoter (**Supplementary figure 5s**). Transformants carrying pEM13 were verified by confocal microscopy by checking expression of Lifeact (**Supplementary figure 6s**).

3.5 Injury-induced conidiation assays

Two methods were used for this assay:

1) Culture plates: Strains were grown on PDB medium with a cellophane sheet placed over a single layer of Whatman 1 filter paper for 40 h in the dark. Mycelium was then damaged with a scalpel (making the same number of lines on the mycelium) or by a cookie mold (pressing the mycelium) and transferred to fresh media and incubated for an additional 48 h in the dark. This method was adequate for treatments where soluble compounds had to be added to the media for a limited time.

2) Solid media plates: Strains were grown on PDA medium for 36 h in the dark. Mycelia were then damaged with a cookie mold.

To evaluate the role of extracellular ATP in the response to injury and the possible affinity compared with other nucleotides, the wild type strain was grown in the dark on a cellophane sheet placed on a single layer of Whatman 1 filter paper for 40 h, as described above. The fungus was then transferred to plates containing ATP, ATPγ-S, ADP, CTP, UTP or GTP at a 0.1 mM concentration (Sigma), or transferred to plates containing 2 units of apyrase and ATP (0.1 mM). To evaluate the role of calcium, the strains were exposed to 15 mM ethylene glycol tetraacetic acid (EGTA) (Sigma) for 15 min. Similarly, to determine the role of ROS the strains were transferred to medium containing 30 mM N-acetylcystein (NAC) for 15 min. In all cases, colonies were then washed with sterile distilled water and transferred to Petri dishes containing fresh PDB medium and incubated for additional 48 h in the dark. Control colonies were treated with 30 mM N-acetylglycine (NAG). Colonies were photographed; conidia collected in sterile water, and quantified using a Neubauer chamber.

3.7 Western Blot Analysis

Fresh mycelia with cellophane sheets were frozen in liquid nitrogen, ground in a mortar and resuspended in Laemmli's SDS/DTT sample buffer without dye and maintained on ice. Samples were further disrupted by vortexing; cell and cellophane debris were removed by centrifugation (12.000 rpm) for 2 min. at 4ºC. Protein concentration was determined by using the Bradford assay (Bio-Rad) with BSA as a standard. Equivalent amounts of protein (40 µg) were used for immunoblotting and analyzed by 12% SDS-PAGE followed by electro-blotting onto polyvinyl difluoride (PVDF) (Inmobilon®-P) membranes (Milipore, Billerica, MA). The membrane was blocked with 5% low fat milk in TBS-Tween, and probed (overnight at 4ºC) with Phospho-p38 MAP Kinase (Thr180/Tyr182) rabbit monoclonal antibody or Hog1 (y-215) polyclonal antibody (Santa Cruz Biotechnology, CA) to detect phosphorylated and total Tmk3. Phospho-p42/p44 MAP Kinase (Thr202/Tyr204) polyclonal antibody or p42/p44 MAP Kinase rabbit monoclonal antibodies (Cell Signaling Technology, Beverly, MA) were used to detect phosphorylated and total Tmk1, as indicated. The blots for Tmk-P and Total Tmk were run in two separate gels using the same sample and then each blot probed with the corresponding antibody. Horse Radish Peroxidase-conjugated secondary antibodies (Promega, Madison, WI) and Super Signal West Pico Chemiluminescent Substrate (Pierce-search) (Thermo Scientific, Rockford, IL) were used for detection.

3.8 ROS Detection Assays

Superoxide detection was performed as described by (Lara-Ortiz et al., 2003) with slight modifications. The *Δnox1, Δnox2*, *ΔnoxR* mutants and the WT strain were grown on cellophane and filter paper in plates containing PDB for 40 h. The filter papers with the fungus were washed with sterile water and transferred to plates with or without 0.3 mM nitroblue tetrazolium chloride (NBT) (Sigma) aqueous solution, and incubated for 30 min in the dark at 27 °C. Samples were photographed under an Olympus IX71 inverted microscope.

3.9 Light and fluorescence microscopy

For light or fluorescence microscopy, hyphae were observed on agar blocks or microcultures containing either Vogel's MM or water-agar, as indicated. To stain cytoplasmic components lactophenol cotton blue was added to mycelia. Mycelia were examined by bright-field or differential interference contrast (DIC) with a Leica CTR6000-B microscope. Fluorescence microscopy was performed with an EGFP filter (488nm) 60X Plan apochromatic, oil immersion (1.42 A.N.) confocal microscope Olympus Corp. with FlouView FV1000 Software and a Stereoscope Zeiss Lumar V12 performed with GFP filter (488nm). Image processing and visualization were done with Image J. For time courses of hyphal injury, hyphae were cut with a scalpel and visualized with a confocal microscope Olympus FV1000, FluoView™ software.

3.9 Regeneration assay

To evaluate regeneration, colonies of *T. atroviride* (IMI204060), and MAPKs (*Δtmk1*, *Δtmk3*) and NADPH oxidase mutants (*Δnox1*, *Δnox2*, *ΔnoxR*) were grown on minimal medium for visualization of isolated regenerated hyphae; sucrose 1.5% and 1.5% agar medium on slides of glass (Corning®), incubated for 48 h at 27ºC. Then, strains were exposed to 10 mM 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetrapotassium salt, cell impermeant (BAPTA) (Life Technologies®) or 30 mM N-acetyl cysteine (NAC, Sigma®) or 30 mM N-acetyl glycine (NAG, Sigma®) or treated with 2 units of apyrase (Sigma®) for 15 min; the same strains without treatment were used as controls. Mycelium was then damaged with a scalpel and incubated for 5 h. Finally, mycelia were stained with lactophenol cotton blue for 10 min. Mycelium was observed with a 40x objective under a Leica CTR6000-B microscope and photographed. Quantification of regeneration capacity was represented in bars with the mean of regenerated hyphae using a statistical analysis described below.

3.10 Statistical analysis

The program Graphpad Prism version 6 was used for statistical analysis and constructing graphs. All error bars indicate Standard Error of the Mean (SEM). A one-way ANOVA test or t-test followed by Bonferroni multiple-comparison test was used.

3.11 Analysis of $[Ca^{2+}]_c$ Dynamics following mycelial injury

To visualize the calcium signatures on hyphae using Live cell imaging, colonies of *T. atroviride* carrying the calcium sensor GCamP6::GFP (pEM12) were grown on MMV for 36 h on glass slides (Corning®). The hyphae were damaged using a scalpel and visualized with a confocal microscope (Olympus Corp.) with FluoView FV1000 Software or Stereoscope Zeiss Lumar V12. To determine the calcium flux, the fungus was treated with different calcium channel inhibitors; 5 mM verapamil or 100 µM Dantrolene and 10 mM 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N' -tetraacetic acid) tetrapotassium Salt (BAPTA) (Life Technologies®) for 15 min. After that, the colonies were damaged with a scalpel and visualized with a confocal microscope. To analyze the role of ROS or the role of eATP in the promotion of calcium fluxes, the fungus was treated with 30 mM N-acetyl cysteine (NAC, Sigma®) or 2 units of apyrase (Sigma®) for 15 min, and analyzed after damage as mentioned above. Colonies without treatment were used as controls. Fluorescence intensity was quantified per hypha using the image J software.

3.12 RNAseq and differential expression analysis

Mycelia of the *Δtmk1*, *Δtmk3* and WT strains were collected 30 min after mycelial damage and frozen immediately. The WT strain was previously treated with 10 mM BAPTA or 100 μM ATP for 15 min, as indicated. In all cases an injured control without chemical treatment and a control without injury were included, and three biological replicates were analyzed per strain or treatment. Total RNA was extracted with TRIzol (Invitrogen). Libraries for RNAseq were prepared from total RNA for each strain and treatment, using the TruSeq kit protocol (Illumina) and sequenced with the NextSeq 500 sequencer (1x75 format). 75-base-pair-long reads were obtained and mapped to the *T. atroviride* genome V2 using kallisto and quantifying abundances of transcripts (Bray et al., 2016). A differential expression analysis was performed considering as transcribed genes those that had at least three reads per million. This analysis was done using the edgeR package (Robinson et al., 2010). Normalization of the data was performed using the Cox-Reid profile-adjusted likelihood (CR) method. For determining differential expression between the comparisons, the generalized linear model (GLM) likelihood ratio test was used. False discovery rates (FDR) were calculated (Benjamini and Hochberg, 1995) and genes with a FDR < 0.05 and $\log_2$Fold-change ≥ |1| were considered differentially expressed. Subsequently, the differentially expressed genes in each of the comparisons were compared to highlight gene clusters. A clustering analysis using the Pearson correlation model was carried out and these data represented in a heatmap. An enrichment analysis was performed with the hypergeometric distribution method. GO terms with FDR ≤ 0.05 were considered significantly enriched in each comparison.

*4. Results**

4.1 eATP modulates conidiation in response to injury

To Identify components of signaling cascades and injury-induced signal molecules involved in the response to injury, it was evaluated the early responses from three different perspectives: Physiological, biochemical, and transcriptomic analyses.

When a hypha is damaged, release of cytoplasmic content is inevitable and, thus, surrounding healthy cells could recognize its components as danger signals. Accordingly, this work was focused on extracellular signals that might be responsible for injury-induced conidiation in *T. atroviride*, and evaluated extracellular ATP and Ca^{2+} as potential damage signal molecules. For this purpose, *T. atroviride* was incubated with apyrase, an enzyme that hydrolyzes extracellular ATP, or an extracellular Ca^{2+} chelating agent. Degradation of ATP by apyrase or trapping Ca^{2+} with EGTA in colonies that were damaged with a scalpel resulted in strongly reduced conidiation in the wounded area (96% and 98%, respectively), as compared with an injured control (**Figures 4A and 4B**).

Further the effect of adding eATP was analyzed and was found that it strongly induced conidiation in the peripheral region of an undamaged colony (**Figure 5A**). Also was tested the effect of different purine and pyrimidine triphosphate compounds, ADP, and ATPγ-S (a non-hydrolysable analog of ATP). Conidiation was induced by CTP and ATPγ-S, although not to the same extent observed upon application of ATP (**Figures 5A and 5B**). The purine nucleotides ADP and guanosine triphosphate (GTP), as well as the pyrimidine nucleotide UTP had only a minor effect (**Figures 5A and 5B**). These data suggested that energy derived from ATP hydrolysis is not required for the induction of conidiation, and that a putative receptor with higher affinity for ATP than for other nucleotides is required. Finally, the induction of conidiation by eATP appears to be dose-dependent (**Figure 5C**).

*This section contains results published previously

These observations suggested that extracellular ATP (eATP) and Ca^{2+} play a major role in the wound response.

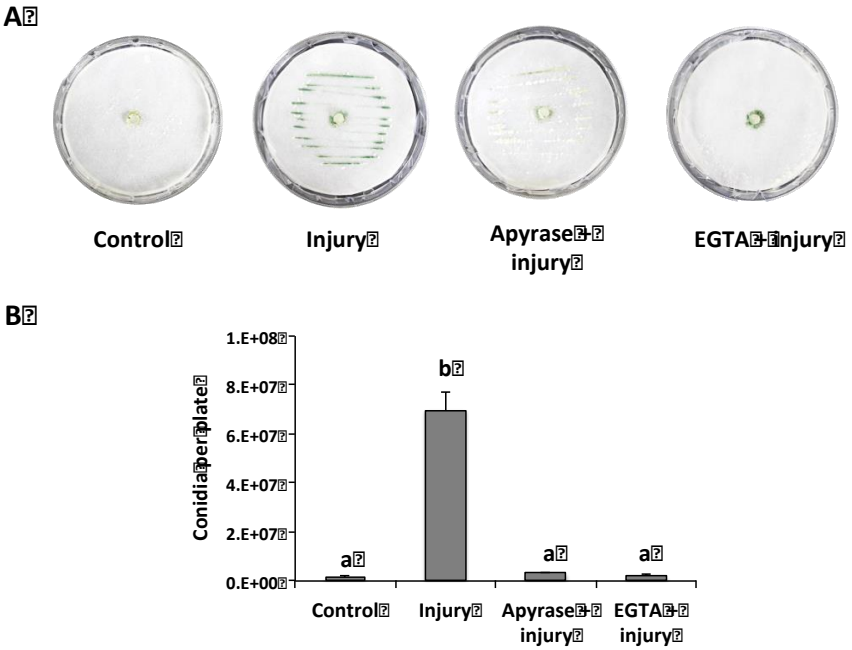


Figure 4. Effect of EGTA and apyrase on injury-induced conidiation. A) Colonies of the fungus were damaged using a scalpel to induce conidiation (visualized as green lines). Prior to damage, apyrase or 15 mM EGTA were added. Photographs were taken 48 hours after injury. An undamaged colony is shown as control. **B)** Quantification of conidia produced after injury. Error bars represent the mean ± SEM of three biological replicas. Bars with different letters indicate treatments that were significantly different ($P < 0.001$). Taken from Medina-Castellanos et al., 2014.

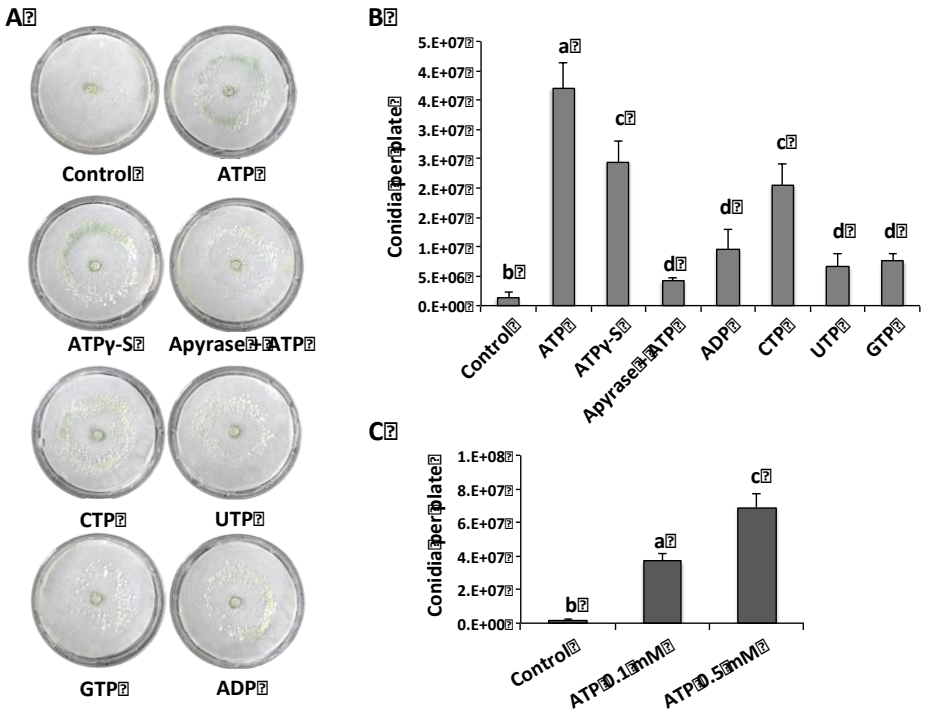


Figure 5. eATP stimulates conidiation. A) Analysis of the WT strain in response to ATP, ATPγS, ADP, CTP, UTP and GTP (0.1 mM), or ATP and 2 units of apyrase. Photographs were taken 48 hours after treatment. An undamaged colony is shown as control. **B)** Quantification of conidia produced after the treatments shown in **A**). **C)** Quantification of conidia produced in response to different ATP concentrations. Error bars represent the mean ± SEM of three biological replicates. Bars with different letters indicate treatments that were significantly different ($P < 0.01$). Taken from Medina-Castellanos et al., 2014.

4.2 eATP signaling promotes Nox1-dependent ROS production

Injury-stimulated Nox1-dependent ROS production is essential for conidiation (Hernández-Oñate et al., 2012). To determine whether eATP could activate NADPH oxidase-dependent ROS production, triggering conidiation. The production of superoxide and conidia in response to eATP were analyzed in the *Δnox1*, *Δnox2* and *ΔnoxR* mutants. Samples of mycelia collected 15 min after eATP induction in the presence of NBT (Nitroblue tetrazolium chloride) were used to detect production of superoxide. After a few minutes of eATP exposure, hyphal tips of the WT and *Δnox2* strains showed the characteristic dark-blue precipitate indicating formazan formation. In contrast, the *Δnox1* and *ΔnoxR* strains failed to produce superoxide at the hyphal tips (**Figure 6A**). To test whether eATP promotes conidiation in the absence of Nox1-dependent ROS, the WT strain was exposed to eATP or injured it in the presence of the antioxidant NAC (N-acetylcysteine). In both cases conidiation was abolished (**Figure 6B**). Similarly, the *Δnox1* and *ΔnoxR* mutants did not conidiate in response to eATP. In contrast,

the *Δnox2* mutant strain conidiated similarly to the WT (**Figure 6B**). These observations indicate that eATP stimulates Nox1-dependent ROS production (acts downstream of eATP). Together these results strongly suggest that eATP is a cell-damage signal that promotes the production of ROS by Nox1. Thus, the activation of putative signaling pathways was explored, such as Tmk1 and Tmk3 MAPKs and determines if it is ROS dependent.

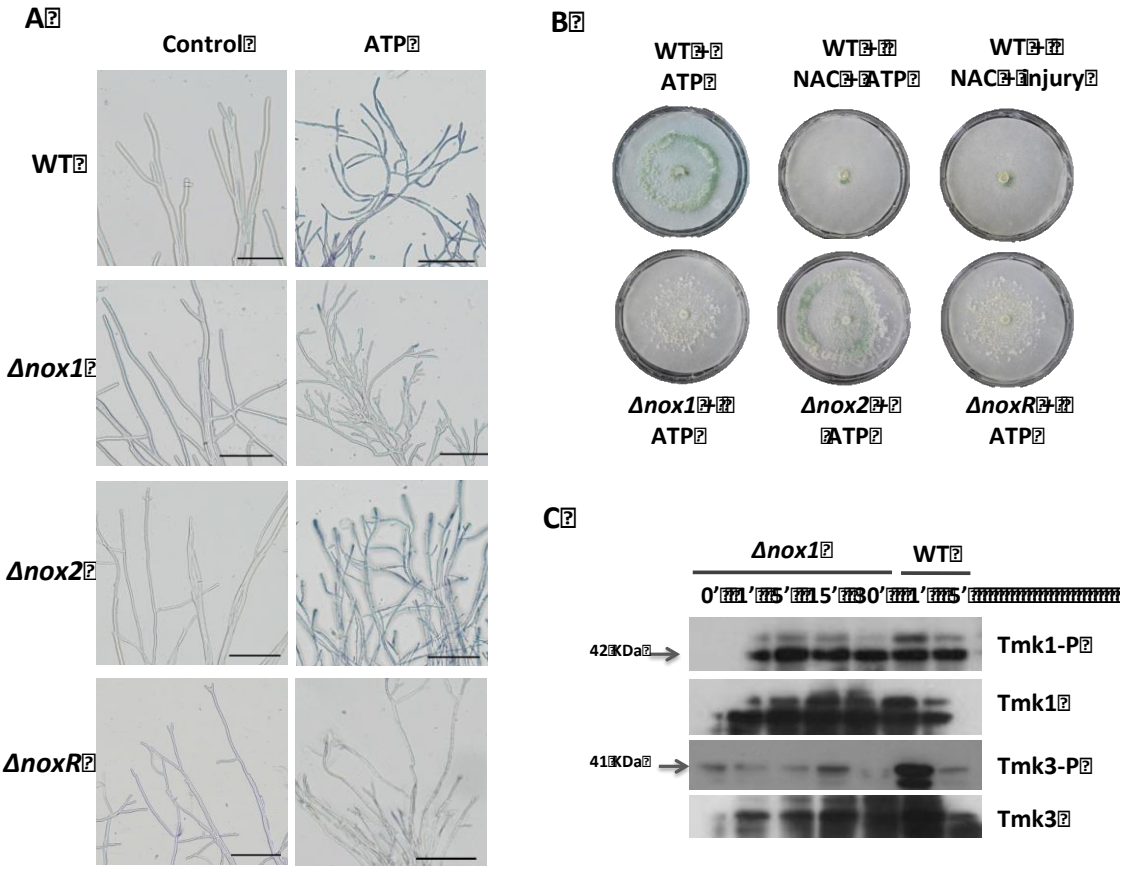


Figure 6. Production of superoxide in response to extracellular ATP. A) Detection of superoxide. WT, *Δnox1, Δnox2* and *ΔnoxR* strains were incubated with ATP (0.1 mM), followed by incubation in a 0.3 mM NBT solution and examined by bright-field microscopy (BF). The blue/purple coloration indicates the production of superoxide (formazan generation). Scale bars = 10 µm. **B)** eATP-induced conidiation. The WT strain was treated with ATP (0.1 mM), or a combination of NAC (60 mM) and ATP, or NAC and injured with a scalpel. The *Δnox1, Δnox2* and *ΔnoxR* mutants were induced with ATP (0.1 mM). Mycelium from an undamaged colony was included as control.

4.3 The Tmk1 and Tmk3 MAPKs are activated in response to injury and eATP

To identify key signaling pathways in the early stage of injury, mutants in two of the MAPKs present: Tmk1 and Tmk3 were evaluated on mycelial growth and conidiation in response to injury. The WT, *Δtmk1* and *Δtmk3* were damaged with a star shaped cookie mold and was observed that the *Δtmk1* strain did not produce aerial mycelia in response to damage, whereas the *Δtmk3* and WT produced aerial mycelia to a similar extent (data not shown). Both *Δtmk1* and *Δtmk3* mutants exhibited a dramatic decrease in conidia production (**Figure 7A**), with reductions of 95% and 80%, respectively (**Figure 7B**). This suggests that transduction of injury related signals leading to conidiation is modulated mainly by MAPK pathways.

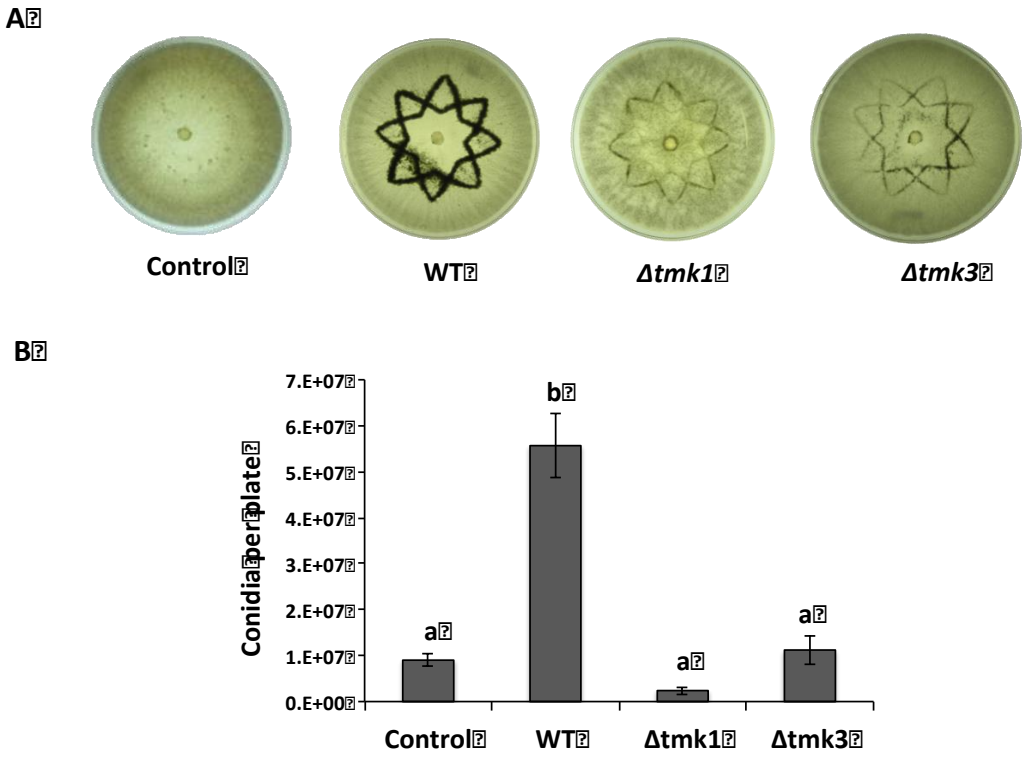


Figure 7. Injury response of the Tmk1 and Tmk3 mutants. A) The WT, *Δtmk1* and *Δtmk3* strains growing on PDA were damaged with a cookie mold, and photographs taken 48 hours later. An undamaged WT strain is shown as control. **B)** Quantification of conidia produced after injury for each strain. Error bars represent the mean ± SEM of three biological replicas. Bars with different letters indicate treatments that were significantly different ($P < 0.001$). Taken from Medina-Castellanos et al., 2014.

To further investigate the activation of MAPK pathways by wounding and eATP, western blots were performed using specific antibodies to detect phosphorylation of Tmk1 and Tmk3. Tmk1 was phosphorylated very rapidly within the first minutes after wounding, but decreasing afterwards; whereas Tmk3 exhibited maximum phosphorylation a minute after injury (**Figure 8A)**. This suggests that Tmk1 plays a sustained role, while Tmk3 participates only during the immediate response. Tmk1 activation appeared to be Nox1 independent, since it was still phosphorylated after injury in the absence of Nox1, while Tmk3 phosphorylation was not observed in the *Δnox1* strain (**Figure 8C**).

Given that injury activates MAPK pathways and that ATP is essential for, and mimics this response, the eATP was evaluate on activation of MAPK pathways. Application of eATP to *T. atroviride* activated both MAPK pathways following similar kinetics to those observed after injury (**Figure 8B**). Using the Tmk1-Phospho and total antibodies, two bands for Tmk1 identification were observed, since the antibodies recognize two MAPKs; Tmk1 (p42) and Tmk2 (p44). However, the antibody showed higher affinity for Tmk1, as shown in figure 4A-B. This result indicates that both stimuli use the Tmk1 and Tmk3 pathways for signaling. Tmk1 and Tmk3 were phosphorylated even when extracellular calcium was chelated by added EGTA (**Figure 8B;** EGTA + injury). Together these results suggest that calcium signaling participates independently of MAPK activation. Therefore, at least three signaling pathways are involved in the wound response, two of them regulated by MAPKs and one involving calcium signaling.

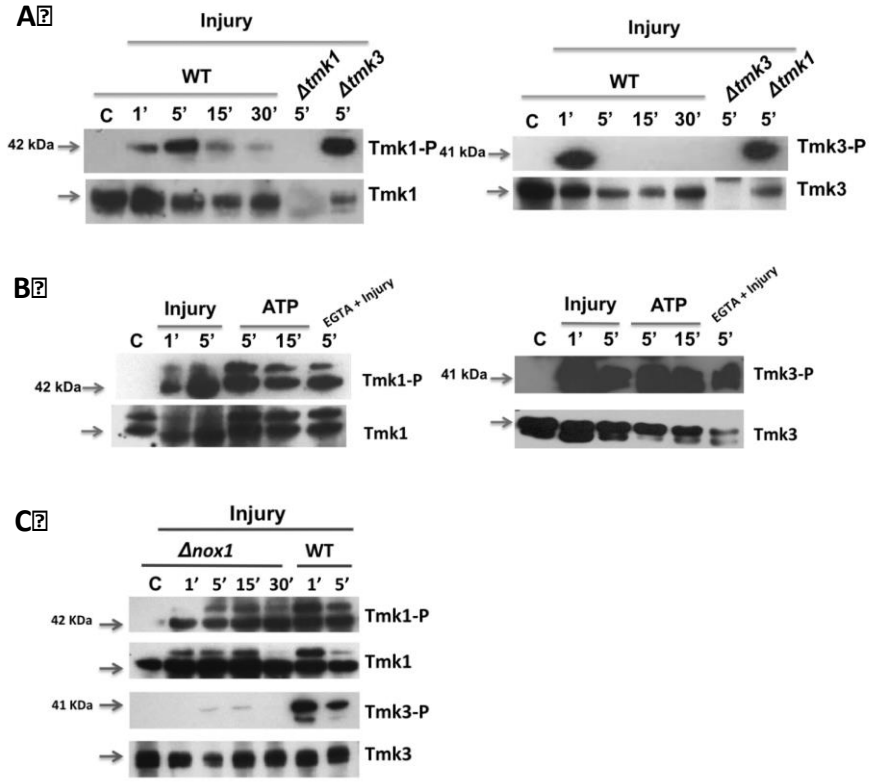


Figure 8. Phosphorylation of TMK1 and TMK3 in response to injury and eATP. **A)** The WT strain was injured and mycelial samples collected at the indicated times. Mycelium from an undamaged colony was included as control. Proteins were extracted, separated by SDS-PAGE, and used for immunoblotting. Blots were probed with anti-Tmk1 (anti-p42/p44) and Tmk1-P (anti-Phospho-p42/p44) antibodies (left panel) or anti-Tmk3 (anti-p38) and Tmk3-P (anti-Phospho-p38) antibodies (right panel). **B)** The WT strain was ATP induced (0.1 mM) or treated with EGTA (15 mM) and mycelial samples collected at the indicated times. Blots were probed as in A). The *Δtmk1* and *Δtmk3* mutants were included as controls. **C)** The *Δnox1* mutant was injured and mycelial samples collected at the indicated times. Blots were probed as in A). Taken from Medina-Castellanos et al., 2014.

4.4 Tmk1 is involved in the regeneration process

According to the previous results, the Tmk1 and Tmk3 pathways are activated in the first minutes after wounding and the mutants *Δtmk1* and *Δtmk3* fail to develop asexual reproduction structures upon injury in *T. atroviride* (Medina-Castellanos et al., 2014). To establish at what stage of the injury-induced conidiation process they participate, hyphal regeneration in the WT, *Δtmk1* and *Δtmk3* strains were analyzed after damage with a scalpel. The regeneration process in fungi refers to the re-initiation of growth through the emergence of a new hypha specifically from the cell adjacent to the damaged one. This

process takes place 1-5 h after injury, depending on the media conditions. Using lactophenol cotton blue it were distinguished between living and dead hyphae. In the WT strain upon injury new hyphae are formed, which emerge from the cell adjacent to the broken one. In this case, the empty (dead) hypha and the emergence of a new one in 65% of the cases were observed (**Fig. 9A & B**). The *Δtmk1* mutant is drastically affected in its regenerative capacity, since in most cases we do not observe the emergence of new hyphae; the empty dead hypha was identified but only in 20% of the cases regenerated. In contrast, the *Δtmk3* mutant developed new hyphae a well as the wt strain **(Fig. 9A),** and showed only a slight decrease in regeneration, 86% hyphae are regenerated (**Fig. 9B).** This suggests that Tmk1 is involved in the early stages of the response to injury, specifically in regeneration, whereas the Tmk3 pathway appears not to be essential for regeneration, even though both MAPKs are phosphorylated in the first minutes after injury and fail to conidiate (Medina-Castellanos et al., 2014).

As described previously, the *Δtmk1* and *Δtmk3* mutants are seriously affected in conidiation. Thus, it is likely that those hyphae that regenerate in the *Δtmk*1 mutant proceed into the conidiophore developmental program without major problem. These results suggest that Tmk1 plays a role in regeneration rather than conidiation, and that Tmk3 plays a role in the later stages of the response to injury, likely in conidiophore development, given that, Tmk3 MAPK is phosphorylated on ROS produced after injury in *T. atroviride* (Medina-Castellanos et al., 2014). In this sense, ROS production has been implicated in differentiation and sexual reproduction in filamentous fungi (summarized in (Scott and Eaton, 2008). Thus, ROS and the Tmk3 pathway play major roles during the late stages of development.

Based on the results described above, it is likely that genes that are no longer responsive to injury in the *Δtmk*1 strain, but still responsive in the *Δtmk*3 mutant are involved in regeneration. Thus, it was identified a set of genes: Injury Responsive genes Tmk1 dependent (IRK1), whose expression changes upon injury in the wt and *Δtmk*3 strains, but that do no longer respond in the *Δtmk*1 mutant. As (IRK1) shown in the Venn diagrams, 157 up-regulated and 133 down-regulated genes were identified, resulting in 285 differentially expressed genes (**Fig. 9C).** The IRK1 set includes up-regulated genes such as a group of hydrophobins (ID 258295, 299543, 258206), thioredoxin reductase (ID 290194), and among

the down-regulated genes, a set encoding ROS-scavenging enzymes, such as a peroxisomal catalase (ID 297668) and a superoxide dismutase (ID 161047). In this regard, Hernandez-Oñate and coworkers had shown that ROS-scavenging enzymes encoding genes are down-regulated 30 min after injury, and that genes involved in lipid metabolism (lipoxygenase, ID 33350; cytochrome p450 monoxygenase, ID 134328) are up-regulated.

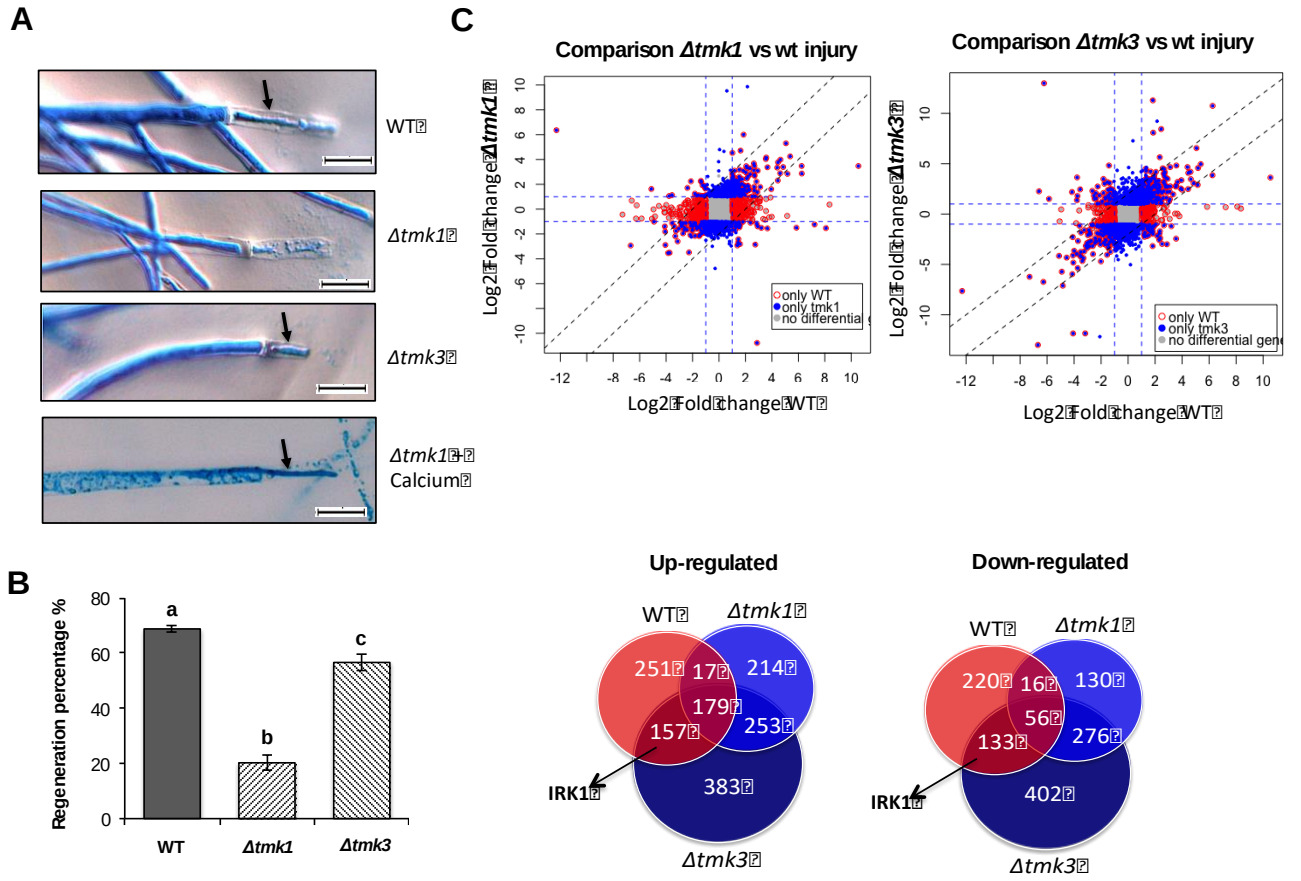


Figure 9. Hyphal regeneration and genic expression profile in MAPKs mutants. A. Microscopic changes observed 1h after injury. Hyphae were stained with lactophenol cotton blue and examined under the light microscope. Black arrows indicate the new hypha. Scale bar= 10 µM B. Quantification of regeneration of strains WT, *Δtmk1*, *Δtmk3.* Mycelia was cut with a scalpel and incubated for 5 h. Error bars represent the standard error of the mean ± SEM of three biological replicates, n=150, different letters indicate treatments that were significantly different $P < 0.01$. C. Plot of the WT vs *Δtmk1* and *Δtmk3* by log2FC, the positive and negative values in the Y-axis represent overexpressed and repressed genes, respectively. D. Venn diagram showing the overlap in up-regulated (left) and down-regulated (right) genes that respond in the WT, *Δtmk1* and *Δtmk3*. RIK1, represent Regeneration Genes Tmk1-dependent.

4.5 Reactive Oxygen Species are not essential for hyphal regeneration

Reactive Oxygen Species are involved in cell proliferation and differentiation in diversity of organisms (Lara-Ortiz et al., 2003; Takemoto et al., 2007), and in the case of fungi also in the interaction with plant hosts (Breitenbach et al., 2015). Importantly, it was recently discovered that Nox1 and NoxR are involved in injury-induced conidiation (Hernandez-Onate et al., 2012). Furthermore, as described above, upon injury ROS activates the Tmk3 pathway in *T. atroviride*, leading to the establishment of conidiation, a suitable response to danger, conidiation. To determine if ROS plays a role in hyphal regeneration, it was performed regeneration assays using NADPH oxidase mutants (*Δnox1, Δnox2* and *ΔnoxR*). In all cases was observed new hypha emerging from the living cell adjacent to the broken one (**Fig. 10A**), and regeneration of cut hyphae was similar to that of the WT strain (aprox. 70% regeneration), with no significant statistical difference (**Fig. 10B, above**). This suggests that NADPH dependent ROS production is not involved in hyphal regeneration. Moreover, the presence of antioxidant molecules such as NAC (N-acetylcystein), does not affect the regeneration process (**Fig. 10B, below).** Altogether, these results suggest that ROS and/or oxidative stress are not involved in the regeneration process. Nevertheless, ROS could participate as signal molecules in the early response to injury as a systemic signal toward nearby hyphae (Hernandez-Onate et al., 2012; Hernandez-Onate and Herrera-Estrella, 2015).

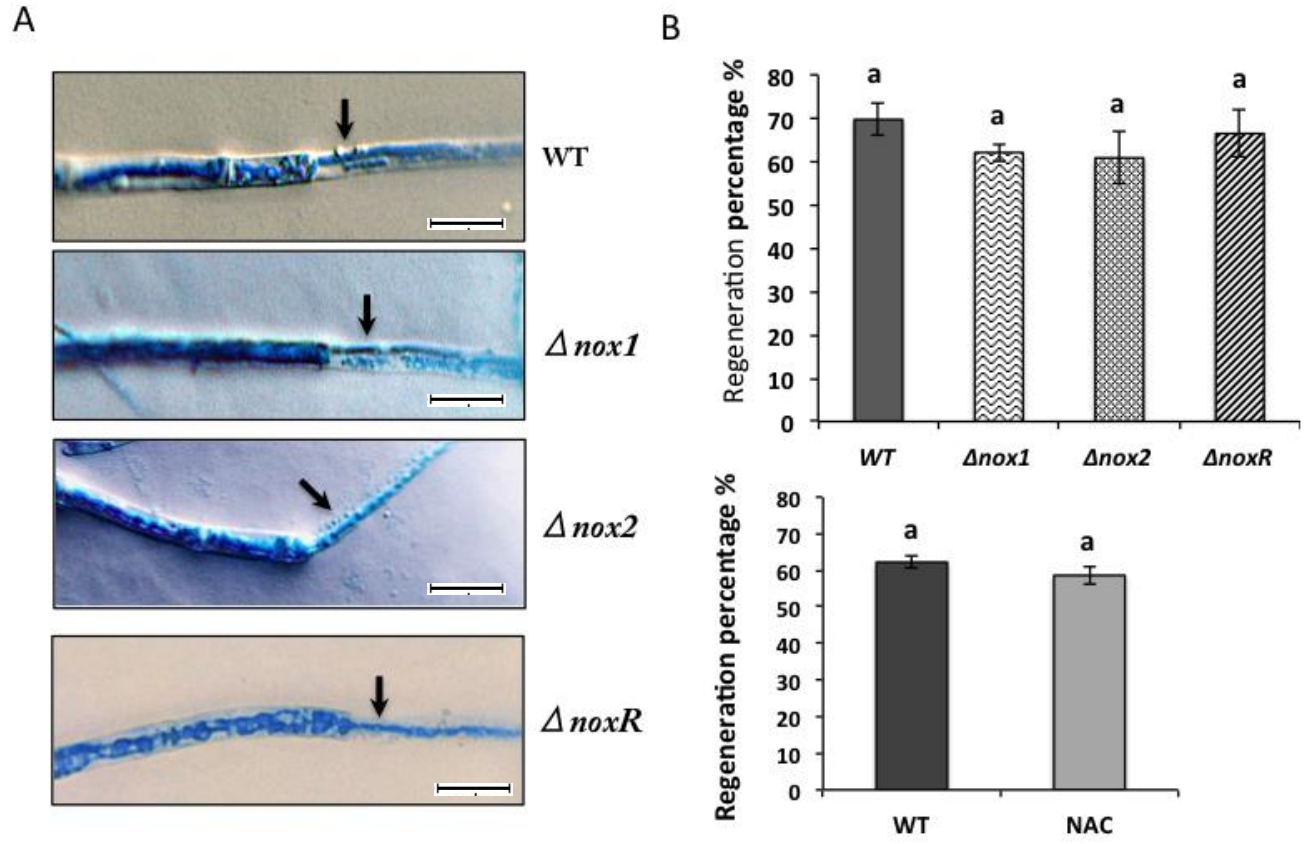


Figure 10. Role of ROS upon hyphae regeneration. A. Microscopic changes observed of WT, *Δnox1, Δnox,2* and *ΔnoxR*, then mycelium was damaged with a scalpel and incubated for 5h. The strains were stained with lactophenol cotton blue and examined under the light microscope. Black arrows indicate the new hyphae. Scale bar= 10 μM **B.** Quantification of regeneration 5h after injury. Error bars represent the standard error of the mean ± SEM of three biological replicates n=150, different letters indicate treatments that were significantly different $P < 0.05$.

4.6 Calcium is essential for hyphal regeneration

According to our results, MAPKs signaling and eATP are key players to establish the injury response. However, there appears to be another, not less important, component, the ion calcium, which has been reported to be essential in many organisms for sealing the injury and for regeneration (Arimura and Maffei, 2010; Cheng et al., 2015) or during the response to different types of stress in fungi (Munoz et al., 2015).

Accordingly, The effect of the lack of calcium using BAPTA (an extracellular chelating agent) was evaluated, on the regeneration process. Under normal conditions, regeneration takes place in the cell adjacent to the damaged one within an hour after damage (**Fig. 11A**), However, hyphae exposed to BAPTA show a significant decrease in regeneration (80%)

and the addition of extracellular calcium restores the phenotype, as shown by the appearance of new, small hyphae re-initiating tip growth (**Fig. 11A-B);** although regeneration is recovered in only 30% of hyphae after adding calcium (**Fig. 11B).** Since it was observed that the lack of calcium results in various cells releasing their cytoplasmic content (not stained with lactophenol cotton blue) instead of one (data not shown), as normally observed in control condition of WT injury, this suggest that calcium is important during the first minutes after injury to seal the septal pore. Interestingly, the inability to regenerate of the WT strain in the absence of calcium is very similar to that observed for the *Δtmk1* strain. Moreover, regeneration of the *Δtmk1* strain is restored by the addition of extracellular calcium (**Fig. 11A-B**). Thus, the TMK1 pathway could regulate components of calcium signaling during the early stages of the injury response.

A transcriptomic analysis of the WT strain subjected to injury in the presence or absence of BAPTA was analyzed. To identify clusters of genes that could be related with regeneration, A clustering analysis and compared groups of differentially expressed genes in the presence and absence of BAPTA were performed, represented by Venn diagrams (**Fig. 11C**).

Calcium-Dependent Genes (CDG); 341 up-regulated genes and 218 down-regulated were identified (**Fig. 11D**); The CDG up-regulated are related with intracellular signaling or cell repair and protection; for example: calmodulin CAMK1 (Id 301592), calreticulin/calnexin (Id 146528), thioredoxin (Id 290194), SNF2 helicase (Id 172559), DNA ligase (Id 83609), phospholipase C (Id 28577), hydrophobins (Id 258295, 299543), and interestingly five ankyrin-repeats containing proteins (Id 316977, 231658, 285335, 173359, 93965); such domains bind proteins involved in: apoptosis, cytoskeletal structure and maintenance of plasma membrane integrity. The down-regulated CDG include a group of Heat shock proteins (Id: 297563, 301737, 299021, 159436), a flavin oxidoreductase NADPH dependent (Id 297928) and a conidiation-specific protein (con-13) (Id 168477) involved in early conidial differentiation in *N. crassa* (Hager and Yanofsky, 1990). The intersection of both Venn diagrams shows Calcium Independent genes (CIG) during the injury response corresponding to 258 up-regulated genes and 207 down-regulated (**Fig. 11C**); CIG up-regulated including genes: cytochrome p450 (Id 36693, 134328), cytochrome b5 (Id

126859), catalase (Id 152289), cysteine-rich secreted protein (Id 44640) and CIG down-regulated genes are related with oxidative stress as well, peroxisomal catalase (Id 297668), superoxide dismutase (Id 161047), polyketide synthase (Id 45973). These results provide further support to the data suggesting a role of Ca^{2+} in the regeneration process.

Together these results suggest that calcium signaling regulates mainly cell repair, cell wall integrity, apoptosis, and cytoskeletal restructuring, and represses at least one gene related with early conidial differentiation genes. Interestingly, calcium signaling does not appear to regulate ROS-scavenging enzymes or enzymes associated with oxidoreduction reactions induced during injury. These results imply that the regulation of genes encoding ROS scavenging enzymes is independent of calcium signaling.

As described above, Ca^{2+} signaling and the TMK1 pathway could be related, since affecting either signaling pathway results in failure to regenerate. The proposed cross-talk between Ca^{2+} signaling and MAPK pathway suggests the existence of regulatory proteins that affect the transcription of common genes. Clustering of GOs-enrichment analysis in injury conditions shows that WT-injury is enriched in DNA metabolism, ribosome biogenesis, nucleobase and cellular nitrogen compounds biogenesis. In contrast, WT-BAPTA versus control and WT-injury do not induce these categories; instead, different categories are enriched, such as organic acid metabolic process, transmembrane transport, oxidation-reduction processes (**Fig. 11D**); likely as a result of stress by absence of calcium and imbalance ionic, thus, the cells have no priority in regeneration, but to stay alive.

Together these results indicate that the Ca^{2+} signaling and the TMK1 pathway participate in the early response to injury involving cell signaling, repair and protection genes.

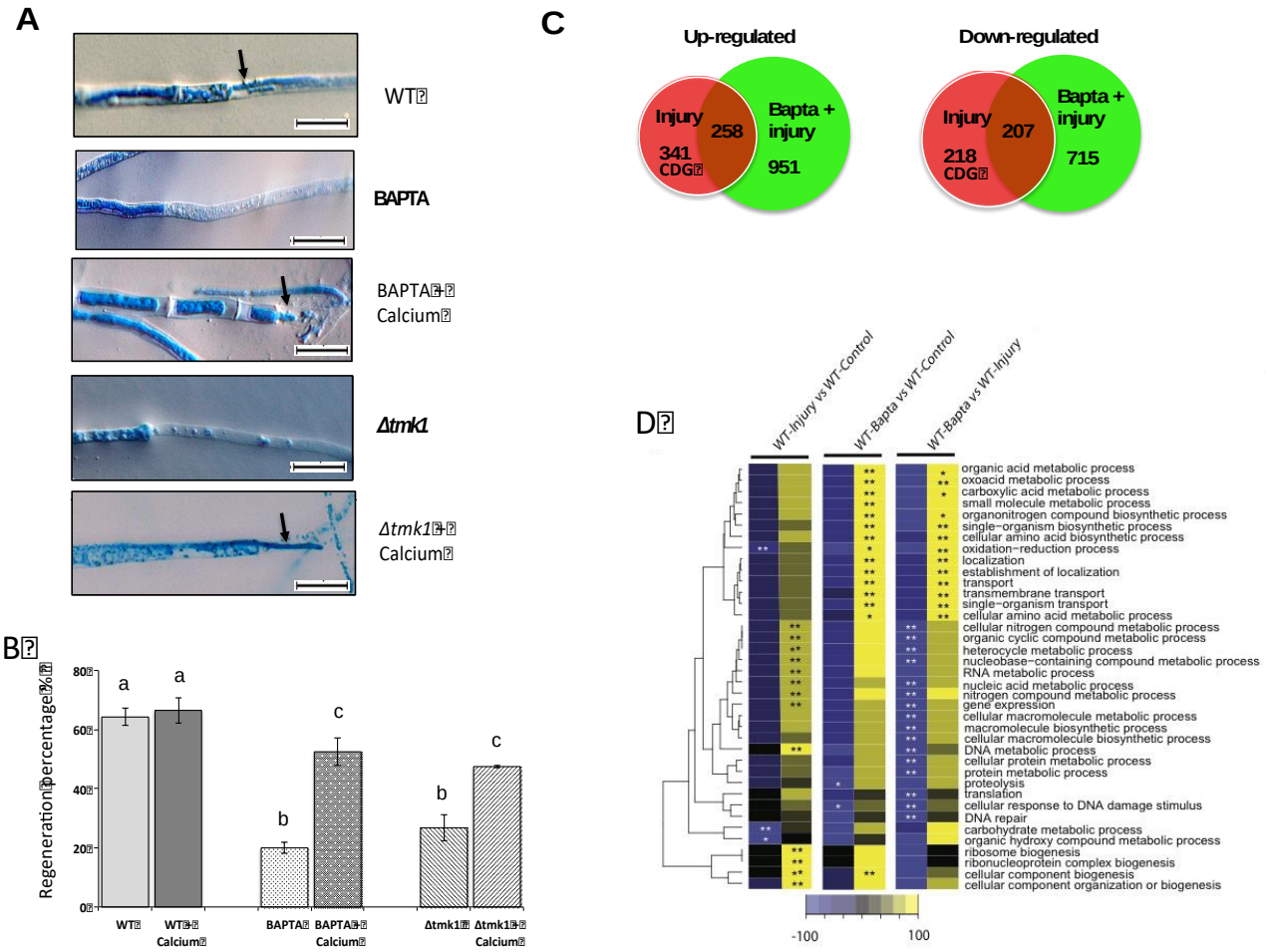


Figure 11. Role of calcium in hyphal regeneration. A. Microscopic changes observed in WT and *Δtmk1* mycelia with or without calcium. WT was grown in presence of 10 mM BAPTA for 15 min; then mycelium was damaged with a scalpel and incubated for 5h. Regeneration of the WT and *Δtmk1* was restored by the addition of 0.02% $CaCl_2$. Both strains were stained with lactophenol cotton blue and examined under the light microscope. Black arrows indicate the new hypha. Scale bar= 10 μM. **B.** Quantification of regeneration 5h after injury. Error bars represent the standard error of the mean ± SEM of three biological replicates n=150, different letters indicate treatments that were significantly different $P < 0.05$. **C.** Venn diagrams showing the overlap between Injury and BAPTA injury, up-regulated and down-regulated. CDG, represents Calcium Dependent genes. **D.** Clustering of GOs-enrichment analysis belonging to Biological Process (FDR <0.01 **; FDR <0.05 *).

4.7 Injury induces a transient increase in $[Ca^{2+}]_c$ in mature vegetative hyphae through a calcium-induced calcium release system

Considering the relevant role of calcium in regeneration, the mechanisms involved in calcium signaling were studied in more detail. First, it was evaluated potential calcium signatures produced at a wound site in *T. atroviride*. Live imaging analysis of wounded cells was performed using the genetically encoded calcium sensor GCamP6 in *T. atroviride*. Live-imaging of calcium influx was obtained following injury of a hyphae. Interestingly, there was a drastic increase in $[Ca^{2+}]_c$, immediately (2 sec) after injury; this effect is a transient spike of cytosolic calcium that decreases over time (**Fig. 12A**).

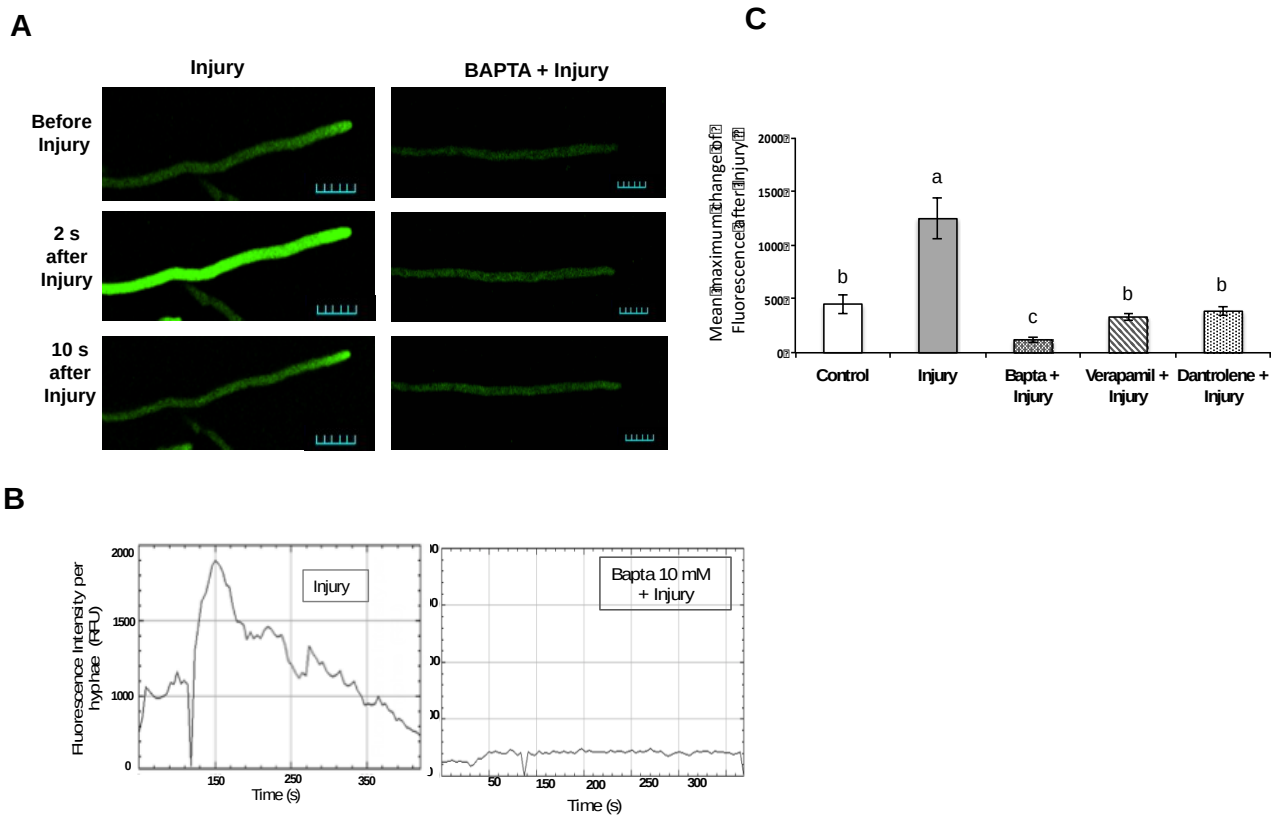


Figure 12. Calcium signature during the response to injury. A. Live cell imaging. The WT strain containing pEM12 was damaged with a scalpel or 10 mM BAPTA for 15 min and then damaged; photos were obtained using time-lapse confocal microscopy. Scale bar = 10µM. **B.** Relative fluorescence per hyphae represented indicated in A versus time. **C.** Bars show the mean maximum change of fluorescence after injury with different treatments calcium inhibitors. WT strain was damaged after 15 min with 10 mM Ca^{2+}-chelator BAPTA empty bar as control without injury. Error bars represent the standard error of the mean ± SEM of three biological replicates, n=150, different letters indicate treatments that were significantly different $P < 0.01$.

The calcium wave observed moves outwards starting from the injured hyphae toward both sides of the mycelium (data not shown). To determine if the source of calcium is intracellular or extracellular, used the extracellular calcium-chelating agent (BAPTA). Treatment with BAPTA 15 min before injury abolished the calcium signature; cytosolic calcium remained low and constant through time (**Fig. 12A**). As it can be seen in the histograms that show the fluorescence intensity per hypha, injury promoted a transient elevation of $[Ca^{2+}]_c$, up to 1800 relative fluorescence units, while BAPTA treatment suppressed this elevation (**Fig. 12B**). Initial low fluorescence intensity is observed preferentially at the hyphal tip in the BAPTA treatment. This reduction is likely because the external free Ca^{2+} is sequestered by the chelating agent and is necessary for promoting small increases of Ca^{2+} during growth. These results suggest that extracellular calcium is necessary to generate the observed calcium signature during the injury response in *T. atroviride.* A mechanism regulated by intracellular calcium pools, which releases calcium into the cytosol and is activated through a calcium influx, known as calcium induced calcium release system has been described in mammals (Endo, 2009; Fabiato, 1983). To test if such a mechanism operates during the response to injury of *T. atroviride*, it was applied the calcium release inhibitors; Verapamil blocks L-type calcium channels, and Dantrolene inhibits Ca^{2+} release from the sarcoplasmic reticulum pool. Both Verapamil and Dantrolene significantly reduced cytosolic calcium increases due to injury (**Fig. 12C**). These observations strongly suggest the participation of a calcium induced calcium release system in the response to injury, in which the calcium influx (extracellular calcium) activates the release Ca^{2+} ions from intracellular pools. Finally, the outcome is an elevation of cytosolic calcium resulting from extracellular calcium influx and mobilization of Ca^{2+} from intracellular pools. This suggests that *T. atroviride* possesses a mechanism based on an electrochemical potential gradient related to that observed in muscle contraction in mammals to sense injury, where voltage activated L-type calcium channels on the membrane surface allow Ca^{2+} influx, and then calcium is released from organelles.

Then, the Ca^{2+} influx was evaluated on ROS production and determines if there was a correlation. The WT strain carrying pEM12 was analyzed in presence of NAC antioxidant and NAG (analog of NAC) as control of NAC. A spike of calcium after injury in absence of

ROS was observed (**Fig. 13**). This suggests that ROS are not involved in producing calcium influxes; consistent with the previous results, in which ROS are not essential for regeneration processes.

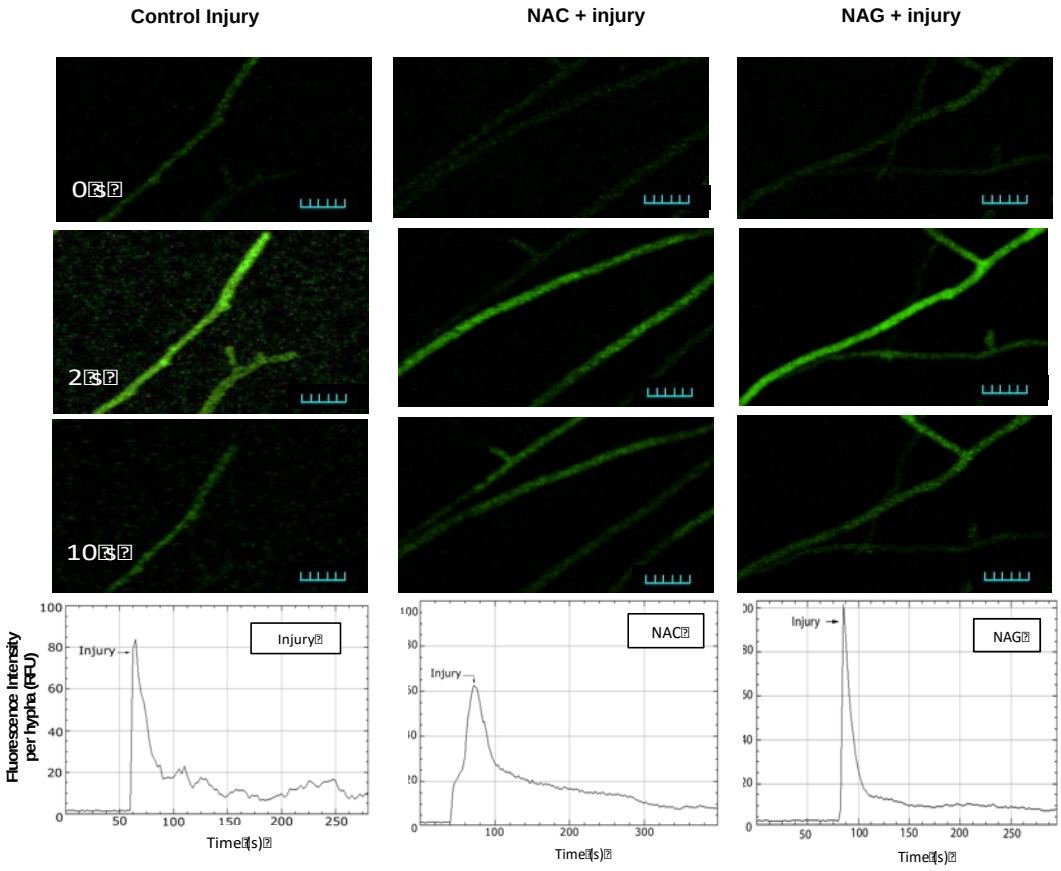


Figure 13. **Ca^{2+} influx is independent of ROS production after injury.** Live cell imaging of the *T. atroviride* wt carrying pEM12, hyphae were treated with 30mM NAC or NAG then damaged. Relative fluorescence per hyphae represented in in live cell imaging versus time. Scale bar = 10µM

Moreover, it was observed that the fungus responds to touch in a similar way than to mycelial injury; increasing $[Ca^{2+}]_c$ but to a much lower level (**Fig. 14)**. Hyphae sense touch or mechanical pressure by displaying an immediate increase in $[Ca^{2+}]_c$, even though when there is no tissue disruption (**Fig. 14**). This suggests that *T. atroviride* possesses sensors that allow perceiving mechanical forces, which trigger calcium signaling. In plants, it has been reported that mechano-sensors are essential to perceive touch, since in mutants

affected in the receptor-like kinase FERONIA, Ca^{2+} signals are abolished when the mutant is exposed to local touch (Shih et al., 2014).

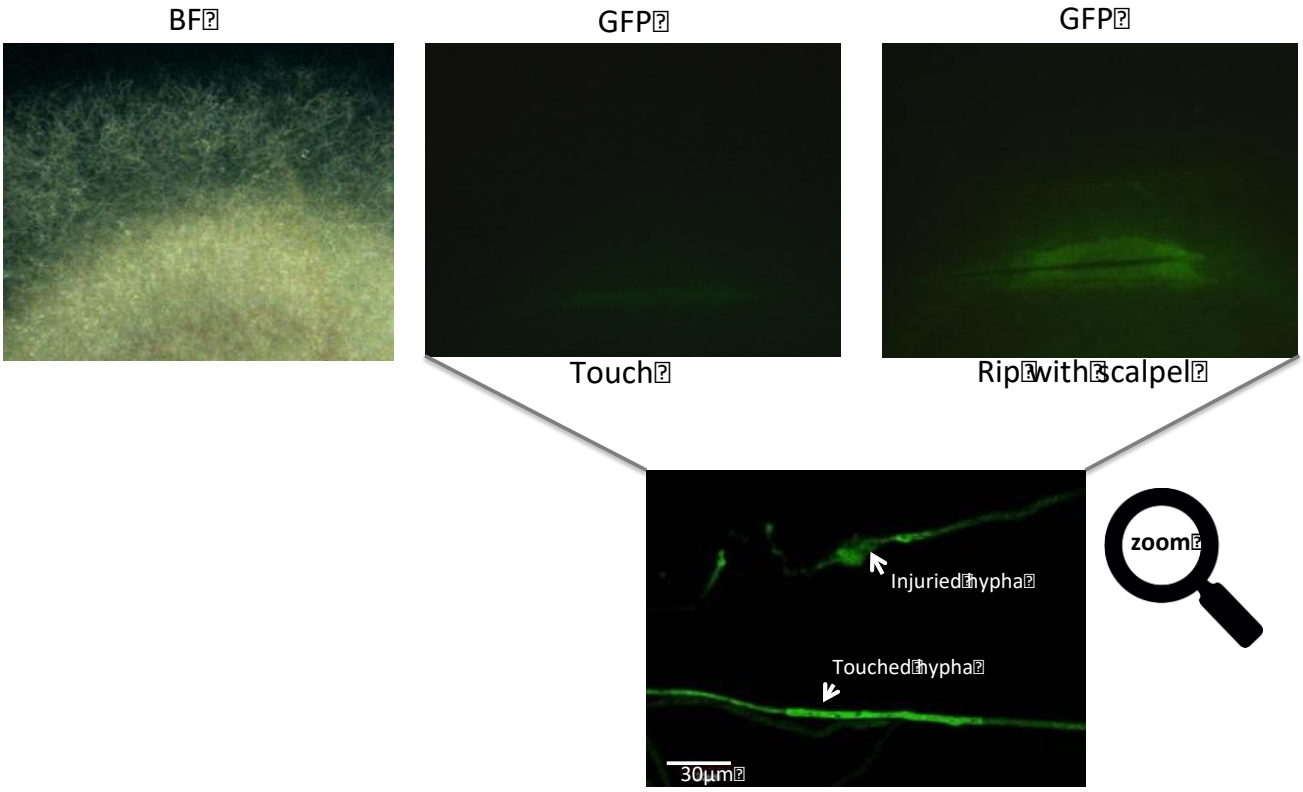


Figure 14. Calcium increase after local touch. The wt strain carrying pEM12 was ripped with a scalpel or touched with the back of a scalpel. Images were obtained in bright field (BF) and with a stereoscope equipped with a GFP filter (top). The bottom image was obtained with a confocal microscope.

4.8 eATP induces a transient increase in $[Ca^{2+}]_c$ in mature vegetative hyphae and is required for hyphal regeneration

The importance of eATP for the response to injury is poorly understood, although, as described above, this molecule activates MAPKs pathways, ROS production and conidiation in *T. atroviride* (Medina-Castellanos et al., 2014). In plants and animals it has been reported that eATP released from disrupted tissue is one of the molecules known as DAMPs that take part in the damage-self recognition mechanism (Heil and Land, 2014; Heil

et al., 2016). To investigate if this molecule triggers a primary signal to sense injury that triggers Ca^{2+} influx, calcium dynamics after injury and regeneration was evaluated by adding extracellular ATP or apyrase, an enzyme that hydrolyses ATP to AMP. Addition of eATP provoked an increase of $[Ca^{2+}]_c$ and the calcium signature produced was very similar to that observed upon injury (**Fig. 15A-B**). When the mycelium was pre-treated with apyrase, the fluorescence signal after injury was strongly reduced, as compared to an untreated (no apyrase) control (**Fig. 15A-B**). This result shows that eATP is necessary for the increase in $[Ca^{2+}]_c$ in the injury response and suggests that it plays a role as a DAMP in *T. atroviride*.

It is known that ATP drives the systemic inflammation and immune responses in mammals (Cauwels et al., 2014), but a direct role in regeneration has not been reported. Therefore, the removal of systemic extracellular ATP was evaluated on regeneration process. As shown in Fig. 13C, damaged hyphae treated with apyrase showed only 26% regeneration, 44% less than untreated hyphae (72% regeneration). Some of the hyphae treated with apyrase exhibited a branching phenotype (**Fig. 15B**), likely as a consequence of the failure to regenerate. Thus, degradation of eATP released from damaged cells decreased the regeneration capacity of the fungus.

Taken together these results suggest that eATP by itself promotes the production of a calcium signature, very similar to that observed upon injury, which could be interpreted as a “danger signal” (a DAMP). Such a signal is transduced within seconds resulting in the increase of $[Ca^{2+}]_c$ through membrane depolarization provoked by calcium influx. eATP would in addition subsequently activate the TmK1 and Tmk3 pathways, leading to a transcriptional response required for regeneration and survival.

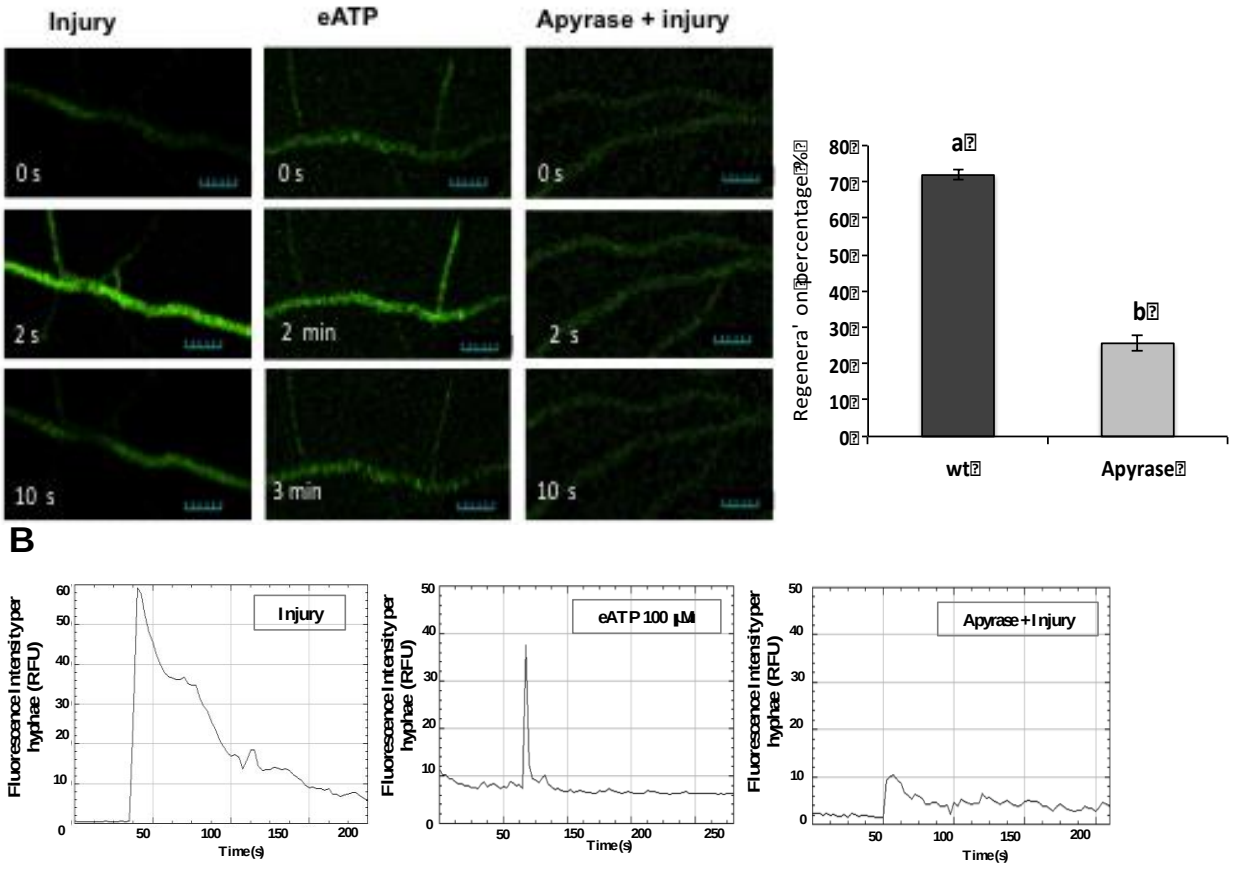


Figure 15. Calcium signature in response to eATP. A. The *T. atroviride* WT containing pEM12 was damaged with a scalpel or with 100 µM eATP or apyrase for 15 min and then damaged; photos were obtained using time-lapse confocal microscopy. Scale bar= 10 µM. **B.** Relative fluorescence per hyphae indicated in A versus time. **C.** Bars show the regeneration percentage % with apyrase treatment.

4.9 Expression of regeneration genes is driven by Calcium signaling and the Tmk1 pathway

Since both Ca^{2+} and Tmk1 indicate to be required for regeneration, it was analyzed sets of genes most likely linked to the regeneration and conidiation processes. For that purpose, clusters were classified using Gene Ontology to identify possible functional enrichments. However, it was not possible to observe any significant enrichment for a specific category. This is not surprising, since it is likely that only a few genes in a pathway or process are essential and sufficient to lead a transcriptional response.

Hierarchical clustering of the differentially expressed genes under all tested conditions showed 9 clusters (**Fig. 16A, heat map)**. Some interesting clusters were selected: cluster 2 represents those genes repressed in wt necessary for the injury response, genes mainly related with cellular carbohydrates, polyol, alditol and alcohol metabolic process. This suggests that in the early stages of the response to injury the fungus needs to limit metabolic chemical reactions derived from the acyclic form of a monosaccharides such as glucose and avoid imbalance between energy intake and energy output. Possibly to save metabolic energy and thus, spend it on re-initiation of growth, these genes are not repressed in *Δtmk1* injury either in presence of BAPTA (**Fig. 16A, box plot**). Moreover, Tmk1 and Ca^{2+} likely regulate these metabolic pathways. When *Δtmk1* or the WT strains exposed to BAPTA were injured, genes belonging to these categories were not repressed. The cluster 8 is interesting, since it includes genes induced only in the injured WT strain and upon ATP treatment, such as lipoxygenase, ankyrin containing proteins and hypothetical proteins (Id 131158, 276604, 301968). These genes are induced by ATP during the response to injury (**Fig. 16A**, box plot), and are transcriptionally regulated by Ca^{2+}, Tmk1 and Tmk3, since they are not induced upon injury in the *Δtmk1* and *Δtmk3*, or the WT strain treated with BAPTA.

In the cluster 9 contains genes most likely involved in regeneration since upon injury these genes are not induced in the *Δtmk1* strain nor in the WT exposed BAPTA (**Fig. 16A**), which do not regenerate. This group of genes includes those encoding proteins related to nucleobase-containing small molecules, nucleoside metabolic process, RNA polyadenylation and lipid modification, suggesting a high replication or RNA processing

activities, as well as chromatin modification, among others (**Fig. 16A**, box plot).

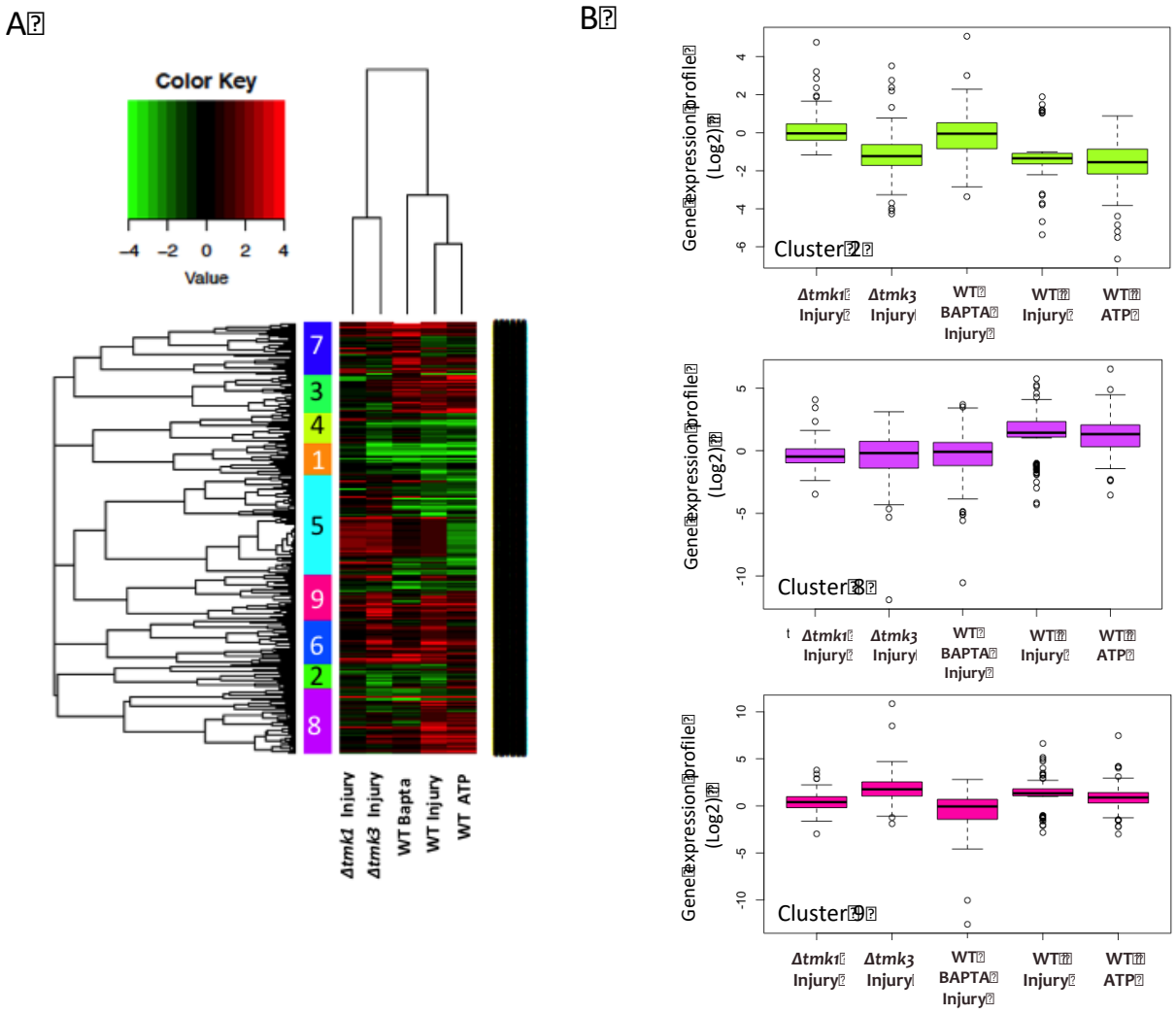


Figure 16. Heatmap and box plots showing co-regulated genes with their expression profiles. **A.** The dendrogram indicates the relationship between gene expression profiles as determined by hierarchical clustering (Pearson correlation). The colored boxes at the left indicate different clusters of genes with similar expression profiles. The color key in the heatmap indicates logFC values ranging from bright red for most up-regulated to bright green for the most down-regulated genes, considering a FDR < 0.01. **B.** The expression pattern for clusters with functional enrichment (clusters 2, 8 and 9) is showing as box plots.

According to previous results, the calcium and Tmk1 dependent signaling pathways orchestrate important mechanisms for hyphal regeneration, therefore, the transcriptional regulation after 30 min could be decisive for lead regeneration and these pathways contribute to activation of components from distinct pathways. To elucidate the transcriptional link between these pathways, it were selected twelve genes involved in lipid metabolism, calcium signaling, antioxidant mechanisms and cell repair and we evaluated the transcripts by counts per million; CAMK1, CrzA (calcium signaling), catalases and thioredoxin reductase (ROS signaling), lipoxygenase, phospholipase A2 (lipid metabolism), DNA/RNA helicase, DNA ligase (DNA double-strand break repair); all these genes have expression profile shared between ATP addition and WT injury (**Fig. 17**).

Genes related to cell cycle were also identified; cell division control protein 54 and cdc-45 are induced in the WT strain upon injury and showed low levels of expression when the fungus had previously been treated with BAPTA, indicating that some cells reinitiate the cell cycle.

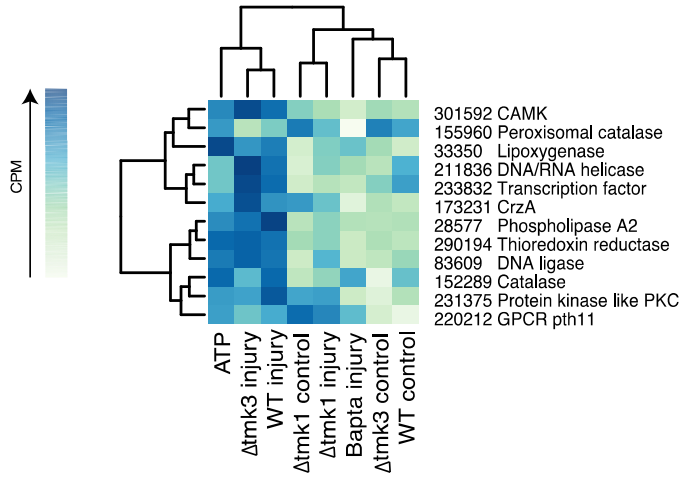


Figure 17. Expression profiles of decisive regeneration program genes. Expression profiles of representative genes of calcium, MAPKs, ROS, and lipid pathways are shown in MAPKs mutants and treatment indicated. The dendrogram indicates the relationship between gene expression profiles as determined by hierarchical clustering (Pearson correlation). The differentially colored boxes at the left indicate different expression between genes profiles. The color key indicates counts per million values ranging from white for most down-regulated to dark blue for the most up-regulated genes, considering a FDR < 0.01.

Interestingly, these pathways are involved in the wound response in plants, insects and mammals (Duran-Flores and Heil, 2014; Heil et al., 2012; Heil and Land, 2014). Among these genes, a lipoxygenase-encoding gene is induced strongly (**Fig. 17**). In this regard, herbivory activates the lipoxygenase (LOX) pathway and LOX-compounds are released rapidly after stress, and are known to serve as “messenger-compounds” in plant–plant communication (Farag and Pare, 2002). This pathway activates multifunctional signaling cascades involving the ethylene and jasmonic acid signaling (Arimura et al., 2002). Interestingly, at least three genes related to lipid signaling are highly induced; lipoxygenase, phospholipase A2, and a putative GPCR-like protein GPCR known as pth11 (**Fig. 17**).

In this analysis, also identified major components of the calcium signaling pathway, such as CAMPK (Calmodulin Protein Kinase) and CrzA. The latter is an ortholog of the *N. crassa* transcription factor Crz1. Interestingly, upon injury the expression of CAMPK and CrzA declines in the *Δtmk1* strain and the WT exposed to BAPTA but not in *Δtmk3*. Suggesting that the TMK1 pathway regulates calcium signaling at the transcriptional level. On the other hand, redox reactions and ROS signaling are induced after injury including a gene encoding a thioredoxin reductase and other enzymes involved in maintaining the redox balance such as catalases (**Fig. 17**). Peroxisomal catalase (Id 155960) and other catalase (Id 152289) are induced in the injured WT strain but reduce the expression in the *Δtmk3* injury (**Fig. 17**). This suggests that TMK3 regulates some genes involved in oxidative stress during the injury response. Finally, we propose a model of regeneration during early stages of the injury response (**Fig. 18**) discussed later.

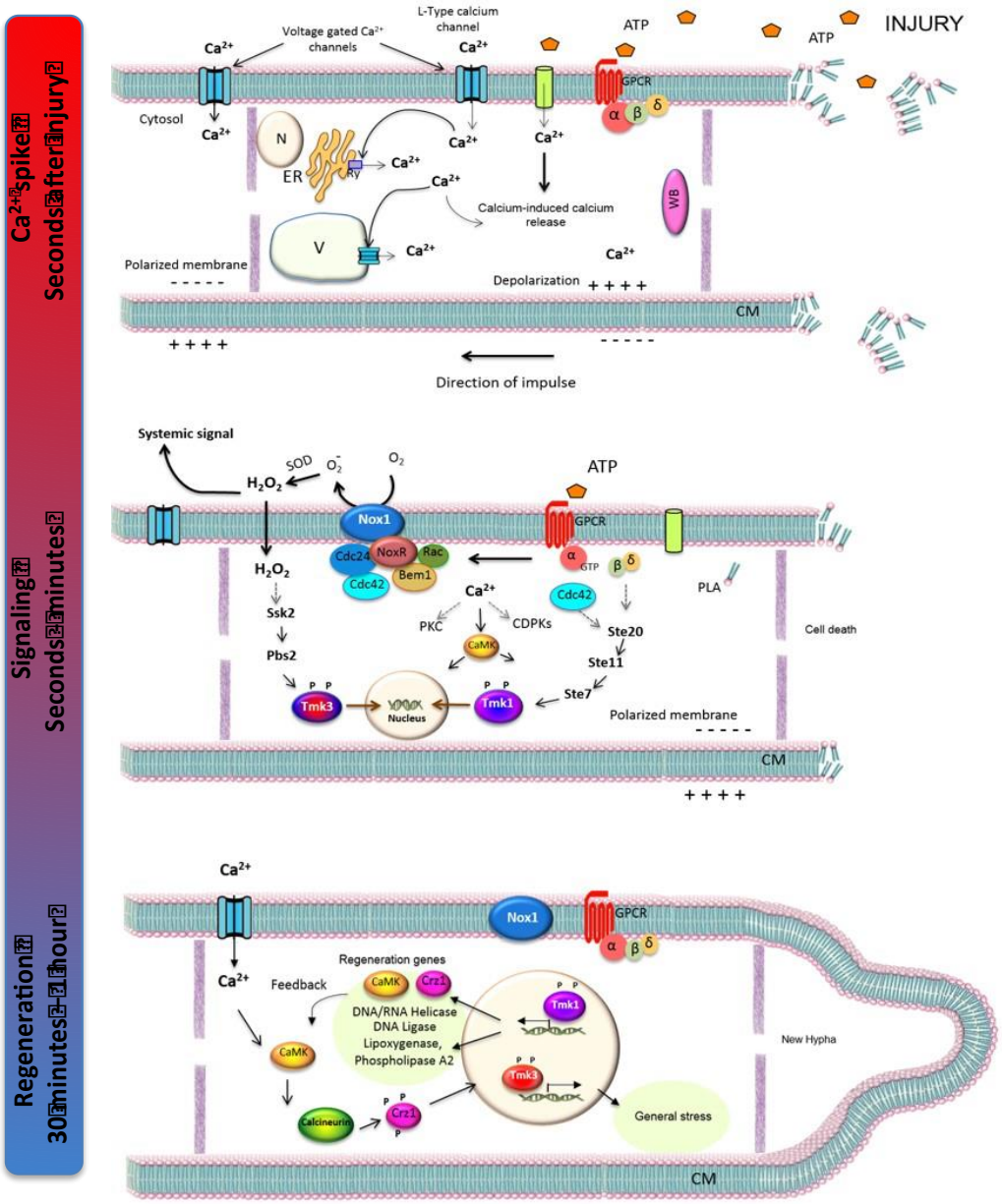


Figure 18.

Early signaling responses to injury in *T. atroviride*. Three early stages of the early response to injury: **1. Ca^{2+} spikes and membrane depolarization**, are detected seconds after injury**,** the increase in $[Ca^{2+}]_c$ depends on L-Type calcium channels and intracellular pools followed by membrane depolarization and sealing the septal pore by Woronin Body (WB). Broken hyphae release ATP as a signal molecule. **2. Signaling events,** ATP is perceived by a putative G protein-coupled receptor (GPCR) activating the Tmk1 and Tmk3 MAPK pathways. Intracellular calcium activates calcium dependent protein kinases, such as CamK1 and other targets. Calcium influx leads to changes in membrane potential (Vm) and could directly activate the Rac GTPase component of the NADPH oxidase complex, generating O_2^-. A superoxide dismutase (Sod) converts O_2^- into H_2O_2 that can diffuse into the cell, activating the Tmk3 MAPK pathway. Phosphorylation of Tmk1 and/or Tmk3 results in the modification of transcriptional profiles. **3. Gene expression and regeneration,** Tmk1 MAPK activation triggers the expression of genes related with regeneration Tmk3 activation triggers to expression genes related with general stress.

5. Discussion

Multicellular fungi respond to wounding by sealing the septa of the cell adjacent to the damaged one by rapidly mobilizing proteins and forming Woronin bodies (Jedd, 2011). In the model fungus *N. crassa*, a single Woronin body is typically translocated to the septal pore nearest to the point of injury and new hyphal tips are generated from this position (Trinci and Collinge, 1974). Recently, a group of septal pore-associated (SPA) proteins, controlling diverse aspects of septal organization associated with Woronin bodies have been identified. SPAs are involved in the cell wounding response in *N. crassa;* in particular, SPA14 has an annexin domain and is likely calcium regulated (Lai et al., 2012). Interestingly, some fungi respond to injury by initiating a morphogenetic change that leads to the formation of different structures, such as sexual fruiting bodies, and conidiophores (Hernandez-Onate et al., 2012; Hernandez-Onate and Herrera-Estrella, 2015). Hernández-Oñate and coworkers (2012) showed that mycelial injury in *T. atroviride* results in the formation of conidiophores that are produced from the newly regenerated hyphae. Their transcriptional analysis suggested that components from distinct pathways: calcium signaling, ROS, Lipid metabolism and protein phosphorylation are involved in the response to injury. In this study, these observations are confirmed and extended, and the signaling mechanism involved in this response are further described and discussed.

A signal molecule was identified that are released to the extracellular medium during injury and that adjacent cells recognize for communication and survival. In plants, eATP is considered a damage signaling molecule, included in a group of molecules known as DAMPs, produced during herbivory, mechanical damage or pathogen attack (Chivasa et al., 2009; Heil et al., 2012; Roux and Steinebrunner, 2007). Interestingly, the data showed that eATP induces conidiation, activates the Tmk1/Tmk3 MAPKs pathways and increases cytosolic calcium promoting hyphal regeneration. This information suggests that eATP is an important signaling molecule that is released from damaged hyphae. Interestingly, in *A. fumigatus* the eATP also induces increases of $[Ca^{2+}]_c$ and the timing of perception of eATP was very much like that

observed in *T. atroviride* (**Fig. 7s** and **Fig. 8s**). Thus, eATP can also be considered a damage signaling molecule in fungi, since which plays a similar role in wound signaling to that reported in triggering immune responses in mammals (Chen and Nunez, 2010; Cordeiro and Jacinto, 2013), fish (Kawate et al., 2009), insects (Moreno-Garcia et al., 2014), algae (Torres et al., 2008) and plants (Chivasa et al., 2009).

Elevations in the concentration of intracellular calcium have also been observed in *Arabidopsis* upon application of eATP (Tanaka et al., 2010) and homeostatic control in signaling of wounding (Tanaka et al., 2014). In this study is observed that injury and eATP cause an increase in intracellular calcium. eATP addition shows dose-dependent trend for injury-induced conidiation. This observation suggests that the fungus senses the concentration of eATP through specific receptor with high affinity for adenosine triphosphate. The dose-dependent response to eATP is probably correlated with the level or extension of the injury, which would in turn result in the production of more conidia to warrant survival of the organism when it suffers from particularly strong damage. eATP would act together with calcium by increasing intracellular calcium to trigger a complete response.

Interestingly, *T. atroviride* also responds to extracellular CTP by forming conidia, consistent with evidence in *Arabidopsis*, where a significant elevation in cytosolic Ca^{2+} could be elicited by the application of exogenous ATP or CTP but not by ITP, TTP or UTP (Choi et al., 2014; Tanaka et al., 2010). In addition GTP, CTP, and UTP (as well as ATP) were found to induce superoxide production in *Arabidopsis* leaves (Song et al., 2006). In this regard, the recognition of nucleotides through purinergic receptors (a family of receptors initially classified according to the relative potency of purine nucleotides to stimulate them), which exhibit different affinities for different nucleotides, is well known in animal systems. Efforts to identify plant ATP receptors through homology of their genomic sequence to animal purinergic receptors failed to find any suitable candidates, but recently the lectin receptor kinase-I.9 (LecRK-I.9) has been shown to perceive extracellular ATP (Choi et al., 2014). In contrast, no single nucleotide receptor has been reported in fungi to date. However, previous results indicate that there must be a nucleotide receptor, with higher affinity for ATP than for other nucleotides.

Although a BLAST based search for homologues of lectin receptor kinases in the *T. atroviride* genome database failed to find any match, the kinase domain of the lectin receptor presents high similarity with a MAPKKK orthologous to yeast Bck1.This protein participates in the protein kinase C signaling pathway that controls cell wall integrity (Lee et al., 1993; Lee and Levin, 1992). In accordance with this, a protein kinase C encoding gene (id 231375) is induced 30 min after injury, suggesting that PKC pathway could participate during the wound response.

Further, in this study is showed that eATP induces Nox1-dependent ROS production, and that ROS activates the Tmk3 but not the Tmk1 pathway. The Tmk1 pathway is likely activated by small GTPases, as proposed in previous reports (Schmoll, 2008). Similarly, Hernández-Oñate et al. (2012) showed in *T. atroviride* that wounding promotes ROS production through Nox1. A recent report in the fungus *Ganoderma lucidum* revealed that Nox-generated ROS increased cytosolic Ca^{2+} levels by activating a plasma membrane Ca^{2+} influx pathway, thereby regulating ganoderic acid biosynthesis and hyphal branching (Mu et al., 2013). In contrast our results indicate that ROS are not essential for increasing $[Ca^{2+}]_c$ after injury neither for regeneration. Although ROS could be important for cell signaling acting as systemic signal at early stages and differentiation of asexual structures during late stages (conidiation), since Hernandez-Oñate (2012) showed that Nox-generated ROS are involved in asexual reproduction after injury. In this regard, it has been established that ROS play essential roles in asexual or sexual development in *Aspergillus nidulans* and *N. crassa* (Lara-Ortíz et al., 2003; Cano-Domínguez et al., 2008), as well as cell signaling roles (Aguirre et al., 2005). In agreement with these observations, *T. atroviride* responds to eATP by developing asexual structures.

Furthermore, in animals, production of ROS by NADPH oxidases or Dual oxidases is crucial for the inflammatory response or activation of systemic defense after wounding (de Oliveira et al., 2014). In plants and animals, Ca^{2+} stimulates NADPH oxidase and Dual oxidase activity through their EF-hand calcium-binding domain, a domain not found in fungal NADPH oxidases. These enzymes in turn produce ROS, which provoke liberation of intracellular Ca^{2+}, likely causing feedback regulation (Niethammer et al., 2009; Wu and Baldwin, 2010; Razzell

et al., 2013). The precise role of Ca^{2+} in the damage response of *Trichoderma* remains to be proven, since calcium released from a broken cell could be detected by neighboring cells as a signal molecule, but it could also serve as a second messenger liberated from intracellular pools or transported across the plasma membrane upon detection of DAMPs. These results suggest that, at least in part, eATP induces conidiation in an extracellular Ca-independent manner, as phosphorylation of Tmk1 and Tmk3 takes place in response to eATP but it occurred in the presence of the Ca-chelating agent, EGTA. All that indicates that more than one signaling pathway may converge in triggering the expression of genes that are required for the wound-induced formation of conidia.

In this sense, one of the earliest signaling events after wound in animals and plants is the activation of MAPKs (Suzuki and Mittler, 2012; Wu and Baldwin, 2010). The first of many reports concerning the involvement of MAPKs in plant-herbivore interactions showed that transcription and activity of WIPK (wound-induced protein kinase), a member of the MAPK subfamily A, increased 1 minute after wounding (Seo et al., 1995). In filamentous fungi, MAPKs play a central role in development and sexual/asexual reproduction (Lara-Rojas et al., 2011; Lichius et al., 2012). In agreement, Tvk1, the *T. virens* ortholog of Tmk1, is involved in conidiation and the activation of genes encoding cell wall proteins (Mendoza-Mendoza et al., 2007). In addition, the ortholog of Tmk3 in *T. harzianum* plays an important role in the oxidative and osmotic stress response (Delgado-Jarana et al., 2006) as well as in asexual development (Lara-Rojas et al., 2011; Lichius et al., 2012). A recent report showed that in *T. atroviride* Tmk3 regulates stress responsive genes and light signaling (Esquivel-Naranjo et al., 2016). Further, Tmk3 and Tmk1 are phosphorylated in a transient manner after injury (Medina-Castellanos et al., 2014). Here we show that Tmk1 and Tmk3, belonging to the filamentous growth/mycoparasitism and osmotic stress pathways, respectively, participate in different stages on the injury response, both necessary to carry out a complete response to the injury. Tmk1 is essential during the early response to injury, since the *Δtmk1* mutant has a strongly reduced capacity to regenerate and shows a transcriptional regulation of genes related to repair and regeneration, in contrast Tmk3 is necessary for conidiation (late response to

injury). Considering that the *Δtmk3* mutant shows levels of regeneration comparable to those of the WT, but fails to produce conidia. In agreement with our results the p38 and JNK MAPK pathways are crucial for axon regeneration in *C. elegans* (Pastuhov et al., 2015). Furthermore, during tissue regeneration and healing in *Drosophila melanogaster*, Grainy head, a transcription factor responsible for epidermal barrier formation and repair, is phosphorylated by ERK1 (Kim and McGinnis, 2011; Ramet et al., 2002). The activation of ERK is also required in mammalian cells for both restoration of damaged tubular epithelial cells and inhibition of fibrosis progression following injury (Jang et al., 2013). Interestingly, Tmk1, the *T. atroviride* ortholog of mammalian ERK1/2 and plant MAP2K1, as well as Tmk3, the ortholog of mammalian MAPK p38 and plant MAPK3, were also activated by wounding (for a review see (Taj et al., 2010).

According to this, the Tmk1 and calcium signaling pathways seem to activate specific genes. Tmk1 and calcium signaling appear to be linked in a particular manner, Tmk1 is activated independently of an extracellular calcium signal, but the Tmk1 pathway induces the transcriptional activation of genes involved in calcium signaling. In animal, yeast and plant cells, a cross-talk between the Ca^{2+}-dependent and MAPK signaling pathways is involved in signaling of abiotic and biotic stress to adapt the cellular metabolism to fast changing environment (Wurzinger et al., 2011).

It has been reported that extracellular calcium is essential to repair disruption of cell membrane by adding membrane components from intracellular vesicles to the cell surface (Suetsugu et al., 2014) and Ca^{2+} release from intracellular pools (Cheng et al., 2015). In accordance with reports in plants and animals, where Ca^{2+} influxes into the cytosol follow the perception of DAMPs (Heil and Land 2014), a calcium signature is evident during the first seconds after injury. This calcium increase would in turn activate calcium dependent signaling pathways, resulting from activation of ion channels or as a second messenger and, could be an alarm signal for adjacent hyphae. Chelating extracellular calcium blocked injury-induced conidiation, which would suggest that calcium plays a key role as second messenger of wounding. The early wound signaling response in animals, including humans (Shabir and

Southgate, 2008; Covian-Nares et al., 2010), and plants (Arimura and Maffei, 2010;

Beneloujaephajri et al., 2013) includes an increase in intracellular calcium and the activation of the calcium signaling machinery. This pathway is also activated by other stimuli. Nelson and coworkers (Nelson et al., 2004) showed that hypo-osmotic shock and external calcium treatment induce transient increase in intracellular calcium in *N. crassa*, *A. niger* and *A. awamori*.

The injury–induced calcium increase is not restricted to the genus *Trichoderma*. Other filamentous fungi like *A. fumigatus,* an important human pathogen, show a similar response. This fungus uses a calcium-signaling pathway increasing intracellular Ca^{2+} just after mycelial damage with scalpel as visualized with a stereoscope (**Fig. 7sA**). The treatment with apyrase and BAPTA prevents the injury–induced increase in $[Ca^{2+}]_c$ compared with control without treatment **Fig. 7sA & B).**

In this study it shows the regulation of at least three genes involved with DNA and RNA replication; DNA helicase are required for nuclear DNA replication, telomere length regulation, and mitochondrial genome integrity in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Pinter et al., 2008). It is known it that yeasts to human, DNA double-strand breaks are repaired by nonhomologous endjoining or homologous integration. The responsible DNA ligase is an enzyme that can repair double-strand or single strand breaks in humans (Pascal et al., 2004). According with this,*T. atroviride* induces a DNA helicase, DNA ligase and genes that suggest cell division and re-start of cell cycle, such as cell cycle control protein 54 and cdc-45. These genes are contained in the GO-enrichment categories and are dependent of calcium (**Fig. 11D**). Interestingly, we also founded genes regulated mainly by Tmk1 and calcium in (cluster 9) (**Fig. 16B**) and likely injury responsive genes (Regeneration and Conidiation) in cluster 8. Genes of blastema formation and tissue differentiation are expressed during regeneration in axolotl and salamanders (McCusker et al., 2015), limb-enriched genes including categories of cell cycle (Monaghan et al., 2012). Our data suggest that cell cycle related genes are regulated by calcium and likely their regulation involves Tmk1, given their similar clusterization in the heat map (**Fig. 16A**). On the other hand, Tmk3 pathway is not involved during regeneration process, since we found a set of genes injury-responsive in the *Δtmk3* mutant, that are also responsive to ATP addition and the WT strain. This set of genes includes calcium transporters, phospholipase C, lipoxygenase, Ca^{2+}/calmodulin-dependent kinase-1 (CAMK-1), phospholipase A2 and CrzA transcriptional

factor. The transcriptional factor CrzA is dephophorylated by the phosphatase calcineurin in the presence of calcium or different types of stress. It has been reported that a CrzA mutant is avirulent and shows decreased conidiation in *A. fumigatus* (Cramer et al., 2008; Soriani et al., 2008). Targets of CrzA were identified in *A. fumigatus.* Among them, several genes involved such as the vacuolar H^+/Ca^{2+}, calcium-translocating P-type ATPase (PMCA-type), calcium-transporting ATPase 1 (PMC1) (Soriani et al., 2010), these genes could be targets of Crz1 in *T. atroviride,* since Crz1 is transcriptionally activated 30 min after injury and genes related with metabolism of calcium are necessary for the regeneration process. Moreover, the activation of calcium signaling during the wound response is not exclusive of *T. atroviride* since *A. fumigatus* also exhibited a cytosolic calcium increase immediately after injury and by the addition of eATP (**Supplementary Figures 7s and 8s)**, therefore this mechanism appears to be conserved in filamentous fungi. We propose the first receptor activated by transcriptional regulation, Pth11 (a GPCR) is induced during injury but it is unlikely an injury specific gene, since this gene is expressed in most of our conditions. Thus, this receptor could mediate a general stress response. It has been reported that Pth11 mediates appressoria formation required for *M. grisea* pathogenicity in rice (DeZwaan et al., 1999) and a variety of GPCRs are closely linked to lipid signaling, such as membrane lipids and GPCRs effectors like phospholipase C (PLC) and protein kinase C (PKC) (Escriba et al., 2007).

In conclusion, it has proposed a model of early signaling responses to injury in *T. atroviride,* divided in three scenarios; fast physiological changes driven by calcium, cell signaling mediated by MAPKs/calcium signaling and regeneration, which involves transcriptional changes (**Fig. 18**). In this work it is described, for the first time in a filamentous fungus, signaling mechanisms and genes involved in the injury response. These are regulated by key elements such as receptors of DAMPs, voltage-dependent calcium channels, intracellular pools driven by calcium induced calcium release system; phosphorylation of MAPKs cascades that leads to transcriptomic activation of decisive genes for regeneration essential for wound healing. In our model proposed (**Fig. 18**), shows eATP as a damage molecule released from wounded hyphae, sensing by putative Pth11-like GPCR at cell membranes of adjacent hyphae. Afterwards, Nox1-dependent ROS production ensues and activates Tmk3. Consecutively, a calcium-induced calcium-released system is activated.

Interestingly, this system is well known in skeletal muscle and neurons (Endo, 2009; Verkhratsky and Shmigol, 1996), this mechanism leads to events of membrane depolarization and electrical signals, which are also observed in plants during herbivory (Mousavi et al., 2013). Reactive oxygen species and TMK3 plays a major role in asexual development but not in the regeneration process after injury.

The RNA-seq analysis allowed establishing and connecting mechanisms involved in the injury response at transcriptional level and determining *T. atroviride* as a good model system to study regeneration processes, given the similarities of mechanisms of regeneration with animal models. In the long term, this will contribute to acquire more knowledge useful in the field of regenerative medicine.

6. Conclusions

- The response to wounding is perceived by specific high-affinity receptors for extracellular ATP.
- Two MAPKs pathway are activated minutes after injury and by eATP addition; namely Tmk1 and Tmk3.
- Tmk3 is activated by Nox1 dependent ROS production.
- Transient calcium influxes take place just after mycelial injury or by touch from the injured region to the outside.
- Tmk1 and calcium signaling regulate hyphal regeneration through transcriptional induction of repair DNA, lipid metabolism and calcium transporter genes.
- Reactive oxygen species are not involved in regeneration processes and do not contribute either in increasing the cytosolic calcium after injury.
- Transcriptomic analyses allowed the identification of a cluster of genes related to the regeneration process controlled by calcium signaling and Tmk1 MAPK.
- The mechanisms of signaling and a set of injury responsive genes are shared in organisms across the tree of life.

7. Perspectives

- Identify the putative ATP receptor by genetic screening.

- Get mutants involved in the cell cycle and determine if the corresponding genes are essential for regenerative processes.

- Determine the implications of a calcium-induced calcium-released system in fungi and advantages over a neuron model.

- Carry out an interaction network analysis to identify injury marker genes responsive to wounding across species.

- Identify targets of Crz1 and associated regulated genes participating in calcium signaling in *T. atroviride*.

8. Supplementary figures

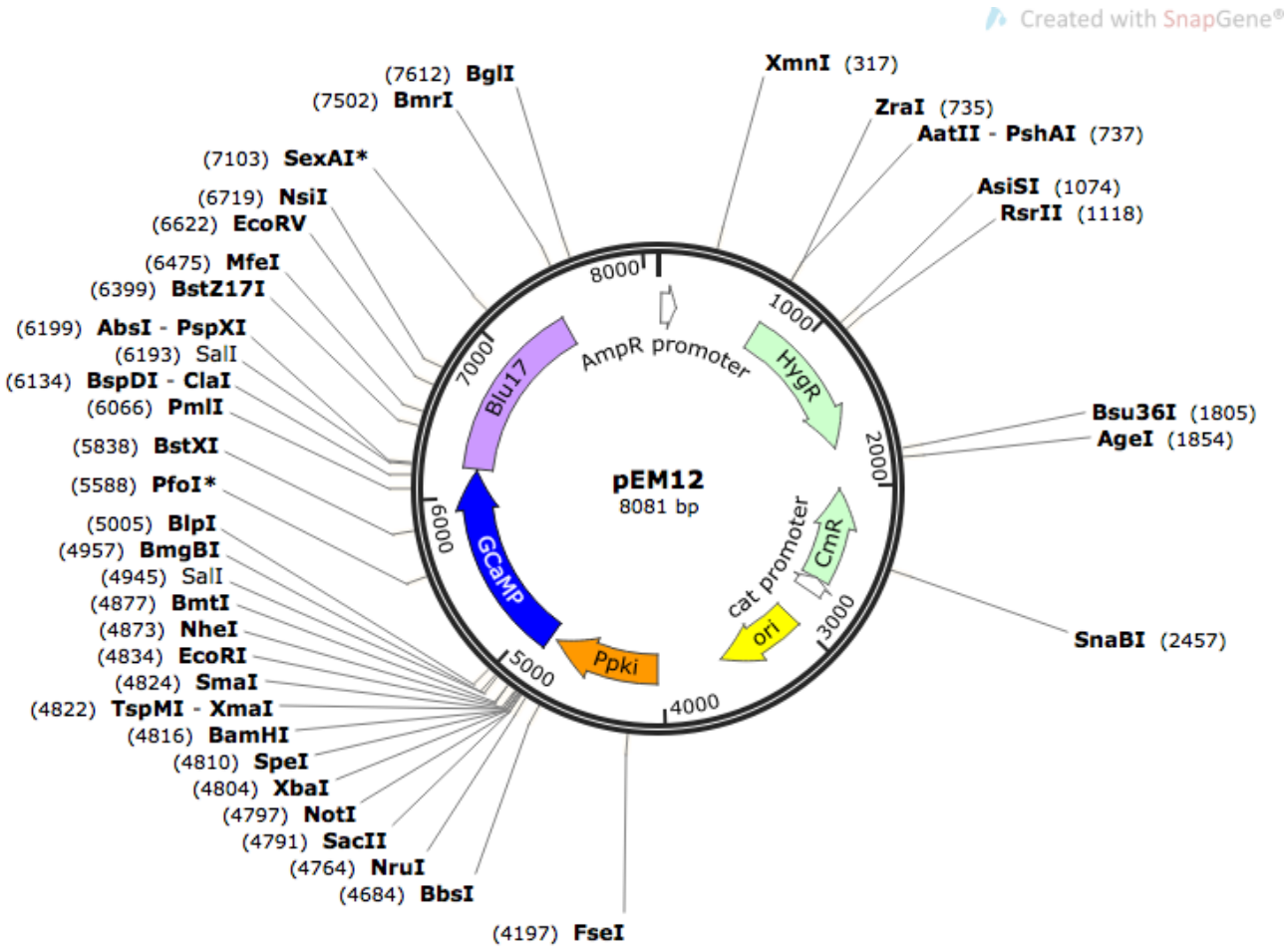


Figure 1s. pEM12 plasmid containing GCamP6. The plasmid is derivate from pUE10 and modify as described in material and methods. Contains a bacterial replication origin (ori), resistance cassette chloramphenicol (CmR), resistance cassette hygromicin (HygR), Piruvate kinase Promoter (Ppki), GCamP6 Open Read Frame and the 3' UTR homologous sequence to T. atroviride *Blu17.*

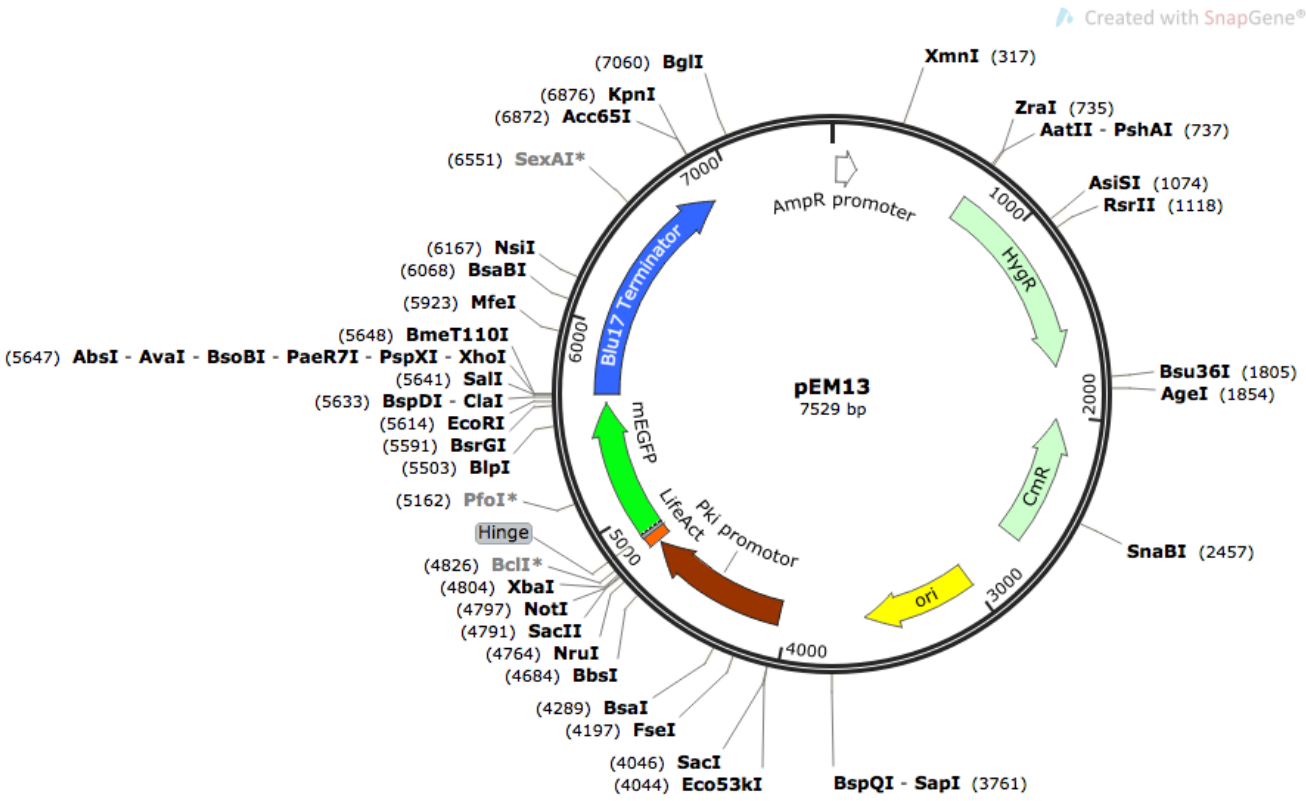
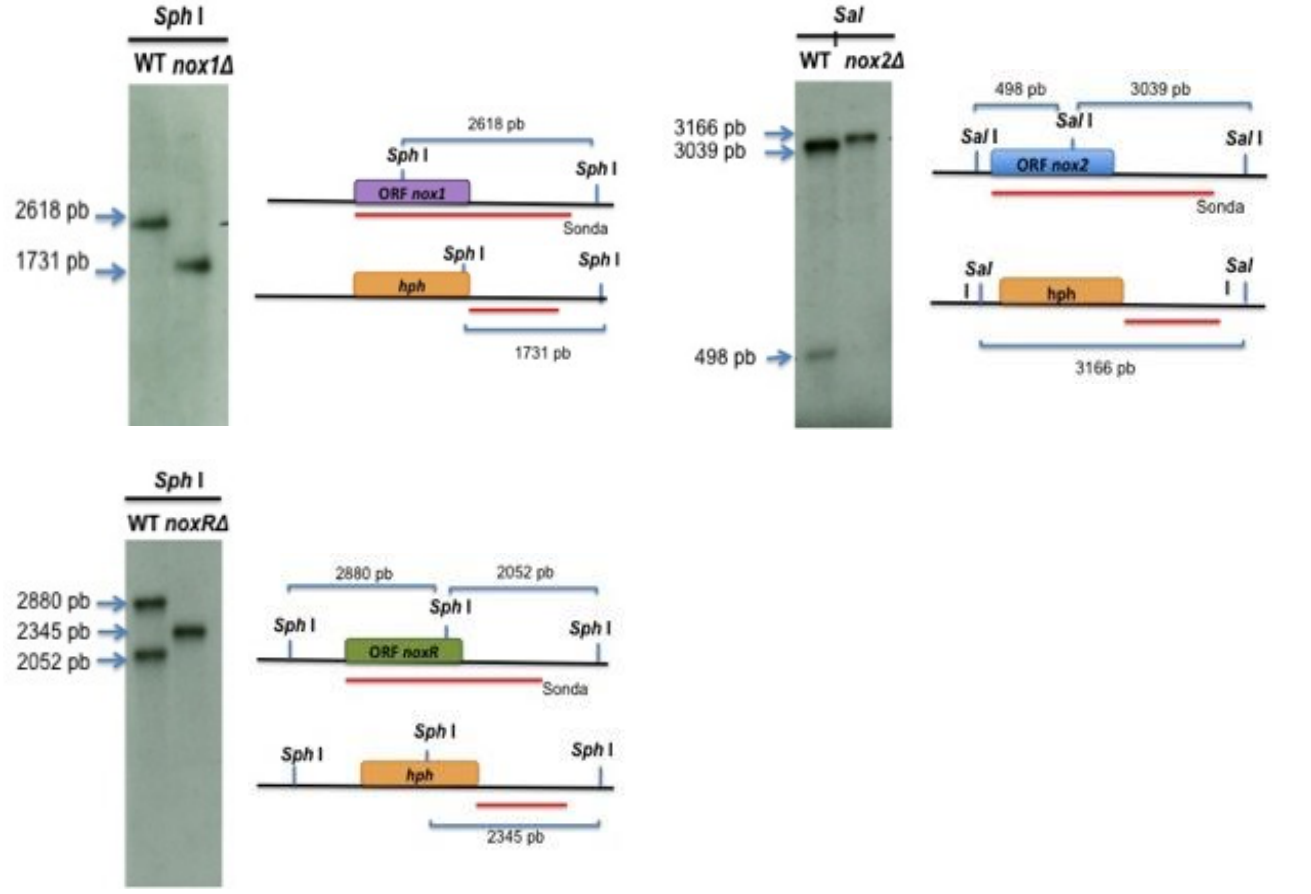
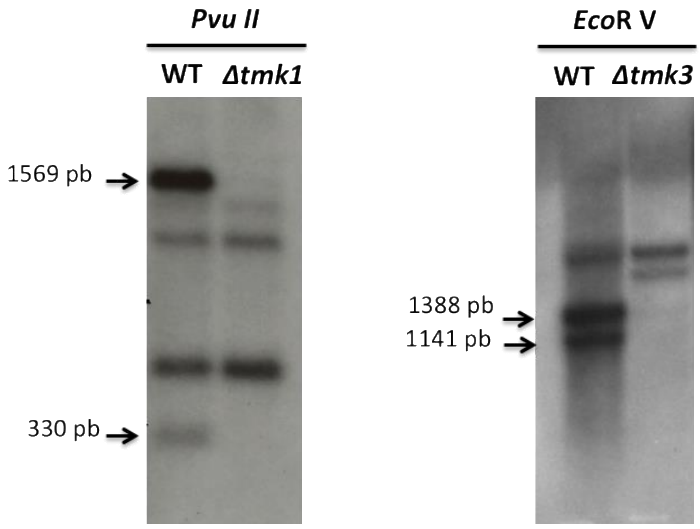


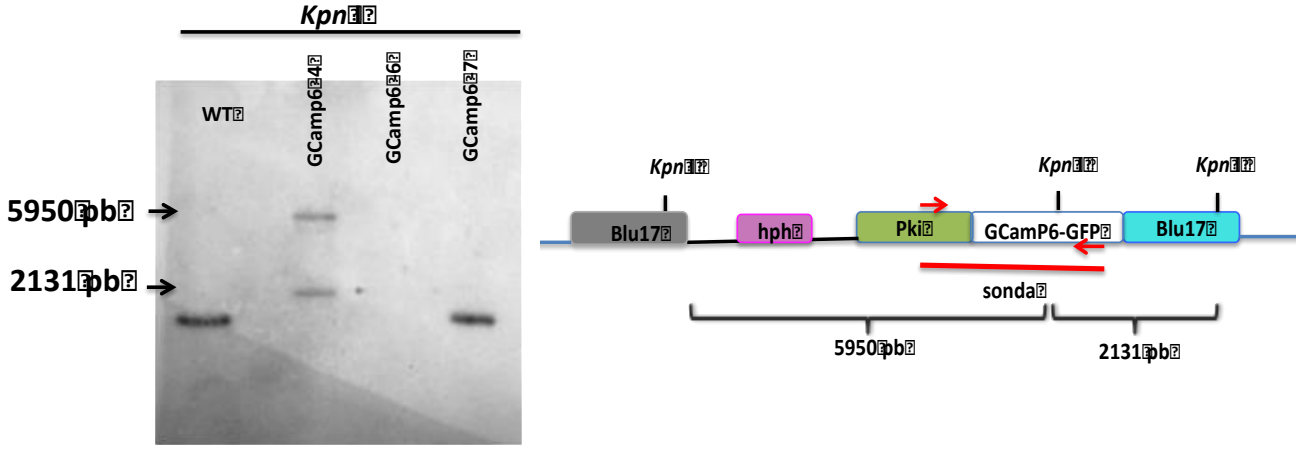
Figure 2s. pEM13 plasmid containing LifeAct GFP . The plasmid is derivate from pUE10 and modify as described in material and methods. Contains a bacterial replication origin (ori), resistance cassette chloramphenicol (CmR), resistance cassette hygromicin (HygR), Piruvate kinase Promoter (Ppki), Lifeact & mEGFP and the 3' UTR homologous sequence to T. atroviride *Blu17.*



Supplementary Figure 3s. Southern blot analysis of *Δnox1, Δnox2* and *ΔnoxR*. Genomic DNA of *Δnox1, Δnox2*, *ΔnoxR* and WT was digested by *Sph* I, *Sal* I and *Sph* I. The probes used to verify the *Δnox1, Δnox2 and ΔnoxR* mutants were a 2.76 kb, a 3.11 kb and a 3.17 kb fragment, respectively included the complete ORF and 1.5 kb at the 3' UTR. Arrows indicate the resulting bands of ORF digestion in each case.

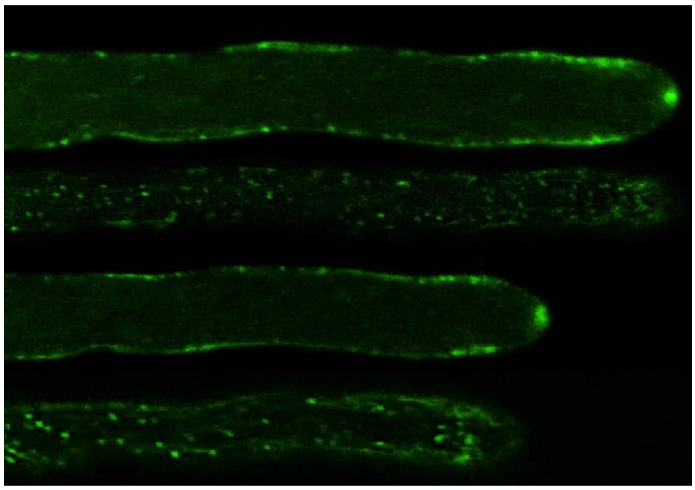


Supplementary Figure 4s. Southern blot analysis of *Δtmk1* and *Δtmk3*. A) Genomic DNA was digested by Pvu II, the probe used to verify the *Δtmk1* mutant (2.9 kb) included the complete ORF and 1.5 kb at the 5' UTR. B) Genomic DNA was digested by EcoR V, the probe used to verify the *Δtmk3* mutant (2.8 kb) included the complete ORF and 1.14 kb at the 5' UTR. Arrows indicate the resulting bands of ORF digestion by Pvu II and EcoR V respectively.

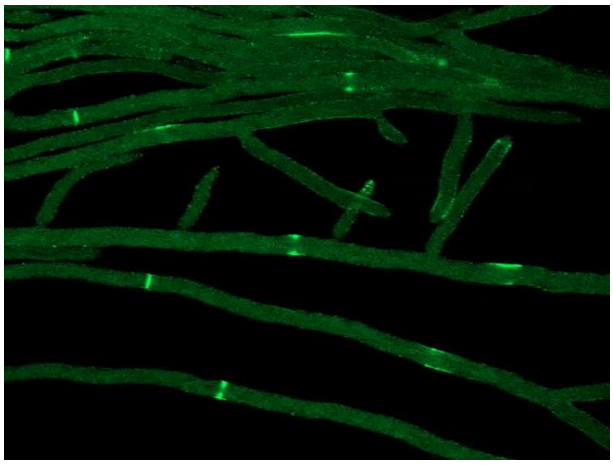


Supplementary figure 5s. Southern blot analysis of *T. atroviride* carrying pEM12. Genomic DNA was digested by *Kpn* I, the probe used to verify the four transformants (1.082 kb) included the complete ORF and 5' UTR. Arrows indicate two resulting bands of ORF digestion by *Kpn*I.

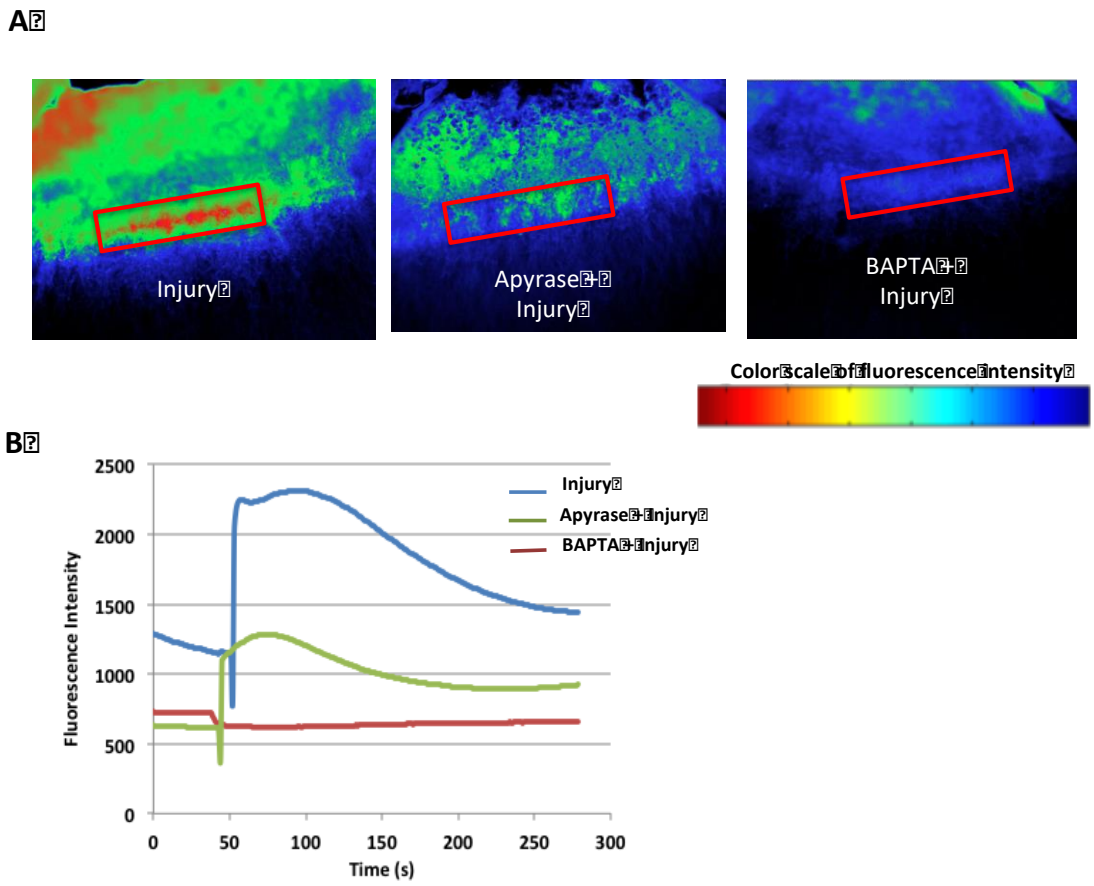
A



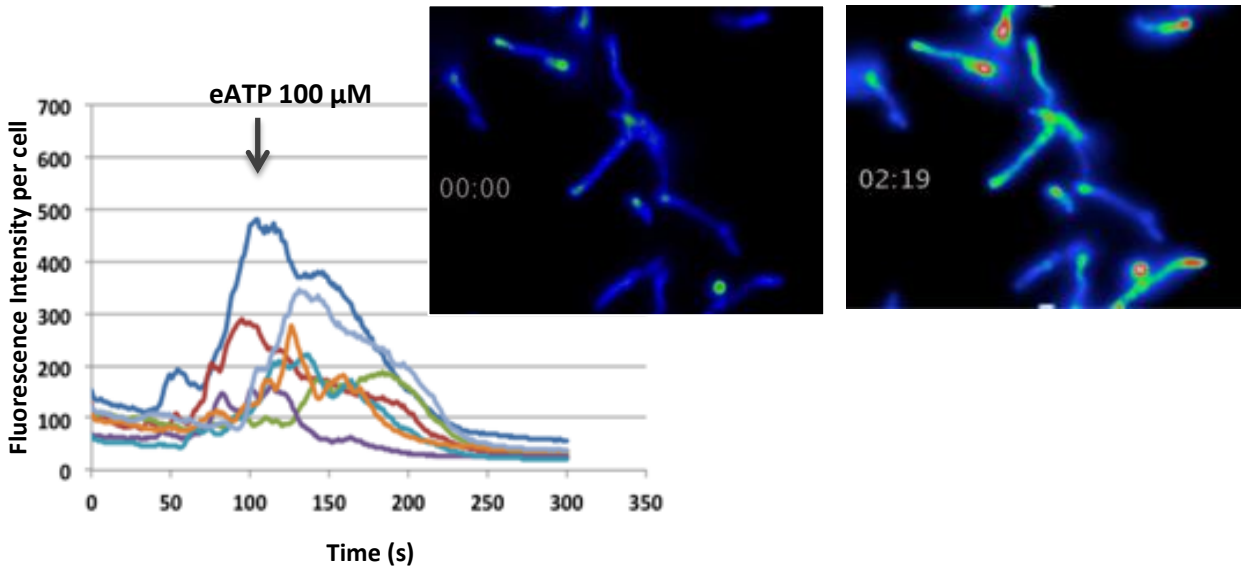
B



Supplementary figure 6s. Actin and septum formation on *T. atroviride.* WT strain containing pEM13 (LifeACT-EGFP) was grown as microcultures and visualized with confocal microscopy. **A.** Actin accumulation on wall cell and apical region (spitzenkorper), actin parches spread on cytoplasm. **B.** Septum formation on mature hyphae, * indicates the initiation of septum development.



Supplementary figure 7s. Cytosolic calcium increase after Injury in *Apergillus fumigatus*. Live cell imaging. **A.** The wt *A. fumigatus* strain carrying the pSK379 construct was injured with a scalpel (left) or exposed to 10 mM BAPTA (right) or apyrase (middle) for 15 min and then damaged; immediately after injury a stack photos were obtained using stereoscope equipped with a GFP filter, the photos show the firs seconds after injury. **B.** The graph show the fluorescence intensity per hyphae indicated in A) versus time.



Supplementary figure 8s. Calcium signature in response to eATP in *A. fumigatus*. Germlings carrying pSK379 were treatment with 100 µM eATP for 15 min and stack photos were obtained using time-lapse inverted microscopy**.** Histogram shows the fluorescence intensity per hyphae versus time.

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10. PUBLISHED ARTICLES