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Thesis

"ROLE OF A SECONDARY METABOLISM GENE CLUSTER IN THE PATHOGENIC INTERACTION BETWEEN *Aspergillus flavus* AND *Zea mays*"

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INTRODUCTION

1. GENERAL INTRODUCTION

The genus *Aspergillus* includes different species with a wide diversity of lifestyle and different pathogenicity mode. *Aspergillus* is one of the best described group mainly because its economic importance and because these fungi are recognized as secondary metabolic producers causing animal and human diseases (S. Amaike and N.P. Keller, 2011).

Aspergillus flavus is studied for its role in producing aflatoxins, secondary metabolites product known to be the most carcinogenic natural substances currently detected in food: that is why they represent a health (causing aspergillosis) and economic issue. *A.flavus* has been studied for years in comparison with *A.fumigatus* but early studies of invasive aspergillosis in non-immunocompromised murine models demonstrated how *A.flavus* is more virulent than almost other *Aspergillus* species; furthermore, *A.flavus* causes a broad range spectrum of disease in humans from cutaneous infection to central nervous system infection (Hedayati et al., 2007).

For all those reasons, it's become very important define the "mycotoxins problem" and control the aflatoxins levels in crop pre-harvest and post-harvest; to do that, it's necessary a global comprehension of cell cycle and infection pathway of *A.flavus* to reduce the damages.

2. *ASPERGILLUS FLAVUS* NRRL 3357

Aspergillus flavus NRRL 3357 (Northen Region Research Laboratory) is a saprophytic soil fungus that infects and contaminates preharvest and postharvest seed crops with aflatoxin, secondary metabolites.

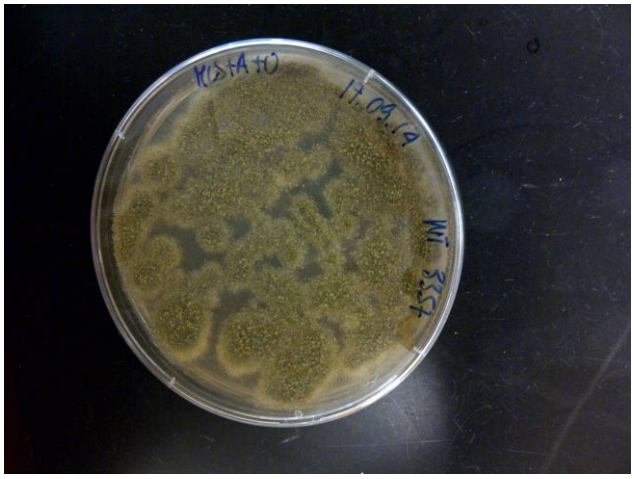


Fig. 1 - *Aspergillus flavus* NLRR 3357

Genus *Aspergillus* includes about 185 species collected in 18 groups; 20 of these are pathogenic to humans; in 1729, the Italian priest and biologist P. A. Micheli was the first to distinguish stalks and spore heads, but only in 1809, Link described the species of *Asperiglus flavus* (Headayati et al., 2007).

Aspergillus Subgenus *Circumdati* Section *Flavi* (also referred to as the '*Aspergillus flavus* group') is divided in two clusters of species. One includes the aflatoxigenic species *A.flavus*, *A.parasiticus* and *A.nomius*, which cause serious problems worldwide in agricultural commodities, and the other includes the non-aflatoxigenic species *A.oryzae*, *A.sojae* and *A.tamarii*, traditionally used for production of fermented foods in Asia (Rodrigues et al., 2007).

Like other *Aspergillus*, *A.flavus* has a worldwide distribution because the atmosphere composition has a great impact for *A.flavus* growth (Gibson et al, 1994). Humidity is the key: it grows better with water activity (w_a) between 0.86 and 0.96. Conidia are able to germinate under more stringent conditions like w_a around 0.65-0.70, values at which bacteria and other molds cannot grow: this allows a very easy colonization during the phases of food storage (as water activity decreases), greatly extending the infection (Vujanovic et al., 2001). The optimum of growing temperature is around 28 - 30°C but fungal development can be observed at lower temperatures ranging from 12° to 48°C (Headayati et al., 2007).

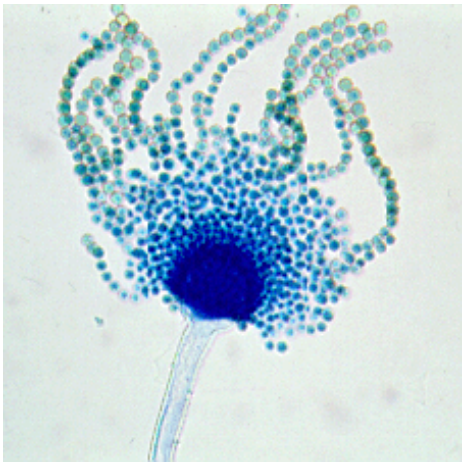


Fig. 2 - Microscopy of *A. flavus* conidia

2.1 GENOME STRUCTURE

A.flavus (NRRL 3357) genome was one of the first filamentous fungi to be completely sequenced; sequencing project started with Gary Payne and Ralph Dean at North Carolina State University and funded by the Microbial Genome Sequencing Project - USDA National

Research Initiative. The USDA/ARS/SRRC in New Orleans, Louisiana provided its Expressed Sequence Tag (EST) genomic database for *A. flavus* as a matching resource towards the complete genomic characterization of *A. flavus*.

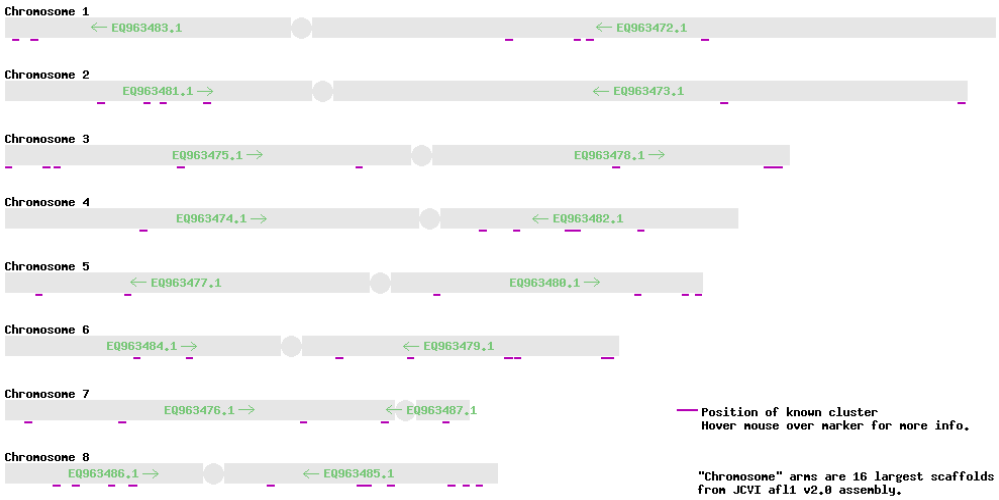


Fig.3 - *A.flavus* chromosomes

The available genome sequence for *A. flavus* provides a powerful resource for research on the biology and evolution of this important plant and animal pathogen: the genome size is about 37Mbp on 8 chromosomes. (http://www.aspergillusflavus.org/genomics/) The genome encodes 14,000 predicted genes; most of them are in common with other *Aspergilli* (Rokas et al., 2007) and the direct consequence is the production of a large genome in *A. flavus*.

2.2 LIFE CYCLE

The success of genus *Aspergillus*, as a group of fungi that are the most abundant in the world, is explain by the fact that they are not very selective respect to their abiotic growth conditions; they can degrade a wide variety of organic molecules (Krijgsheld et al., 2012). In particular, *Aspergillus flavus* is a saprophytic fungus that spends most of its life growing in the soil where it plays an important role as nutrient recycle (Hedayati et al., 2007) and, also, it commonly infects and contaminates major seed crops with aflatoxin, which is highly toxic to humans and animals. The life cycle in agriculture fields can be divided in two stages: the first one is the colonization of debris and the second one is the invasion of seed (Horn, 2007).

During the growth on crops, as peanut, corn, cottonseed and treenuts, it produces aflatoxin to cause diseases (Milićević et al. 2010**;** Visenuo et Mehta, 2015). Under adverse conditions such as dry and poor nutrition, the mycelium congregates to form resistant structures called sclerotia (Yu et al., 2005). Sclerotia are pigmented, compacted aggregates of hyphae, which resist unfavorable environmental conditions and are capable of remaining dormant for very long periods (Wicklow and Shotwell, 1983; Cotty, 1988; Rollins and Dickman, 1998). When growth conditions are favorable, *A.flavus* propagates itself on debris diffusing the conidia throughout the environment; the dormant sclerotia can germinate and sprout additional hyphae or produce conidia, asexual spores, that can be dispersed in the soil and air either (Bennett and Deutsch, 1986).

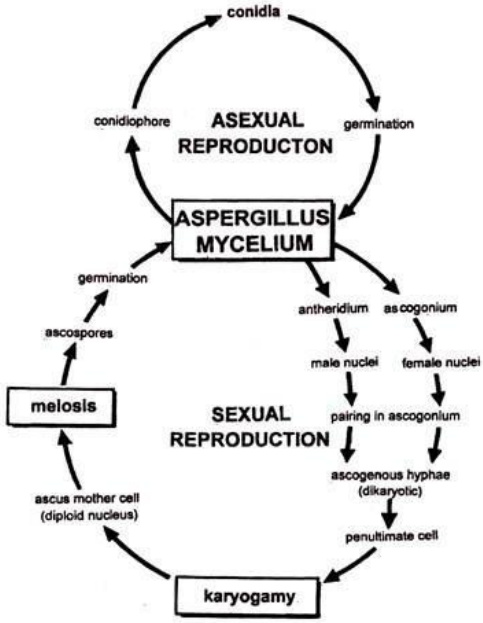


Fig.4 - *A.flavus* life cycle

Recently, *A.flavus* and many other presumed asexual fungi have been found to be capable of sexual reproduction when grown in the dark under nutrient deprived conditions (Ehrlich, 2014).

Besides, *A. flavus* exhibits a sexual reproduction and it consists of a physical contact between two isolates containing opposite mating-type (*MAT*) loci: if one of the mating-type is absent, the fungus cannot reproduce by the sexual way and it reproduces asexually (Fisher and Henk, 2012). Because this fungus can show sexual and asexual reproduction events, it can be exist in heterokaryotic, homokariotyc, recombinant haploid or diploid states and it would be possible that *A.flavus* could maintain one or more states in hyphae or conidia; furthermore, the evidences demonstrate that the major of conidia are homothallic and a small percentage of conidia can harbor heterokaryons or diploid nuclei (Runa et al., 2015). *A.flavus* toxin-producing strains resides in the soil but as an opportunistic saprophytic fungus, it is readily able to colonize most environments whenever there is a rich source of carbon and nitrogen (Ehrilch, 2014); under particular conditions, as after wind or insect dispersal, conidia may infect and produce aflatoxins on susceptible plants. Infected plant and other organic debris within and on soils represent as reservoirs of *A. flavus* for subsequent dispersal to susceptible hosts and/or nonliving food sources (Mehl et al., 2012). During the first phase of contamination, infections are promoted by 1) wounding caused by birds, mammals, insects and mechanically (e.g. hail) or 2) drought stress and 3) elevated

temperatures (Dowd, 1998; Payne & Brown, 1998; Guo et al., 2002). *A.flavus* causes diseases of various agriculture crops such maize, cotton, groundnuts and peanut but the most vulnerable crops are maize (*Zea mays*) (Cardwell & Henry, 2006). Aflatoxin production in corn is extremely variable due, in part, to its sporadic occurrence among ears and among kernels within an ear; fungal structures associated with aflatoxins such as conidial heads and sclerotia likely exhibit a similar sporadic distribution pattern between corn ear and kernel (Horn et al., 2014).

2.3 SECONDARY METABOLISM AND CLUSTER 32

Filamentous fungi produce small bioactive molecules, named secondary metabolites (SMs), exhibiting harmful (e.g., aflatoxins) or beneficial proprieties (e.g., lovastatin) for humankind. Secondary metabolites play important role in fungal ecology as fitness factors but there are not essential for the fungus growth and survival. Those molecules are structurally different with different molecular weight and are encoded by genes organized in clusters present inside the genome, each cluster containing enzymatic genes and often transcriptional factors for compound synthesis (Amaike and Keller, 2011; Keller et al., 2005). Specifically, 56 different clusters (55 clusters identified by Georgianna et al., 2010 and the kojic acid cluster identified by Mauri et al., 2011) were predicted based on the presence of genes encoding four classes of secondary metabolites:

- polyketide synthases (PKSs),
- non ribosomal peptidic synthases NRPs),
- hybrid PKS-NRPs,
- prenyltransferases (PTRs) for terpenoids.

Some of these secondary metabolites protect the fungus against fungivoros predators, like aflavarin that has been show to exhibit anti-insectan activity. (Cary et al., 2015). Last but not least, the most important and best-known secondary metabolites are aflatoxins discovered after the United Kingdom's outbreak of Turkey X disease in 1962, caused by consumption of *A.flavus*-contaminated feed with numerous deaths of turkey poults. Now, in the U.S. alone, *A.flavus* costs 100s of millions USD annually due to market losses of AF contaminated crops (Calvo and Cary, 2015). Furthermore, elevated carbon dioxide levels among with other environmental factors linked to climate change have been show to cause increased expression of genes in the aflatoxin biosynthetic pathway (Satterlee et al., 2016).

Compared to *A.parasiticum* which synthetizes four major AFs (AFB1, AFB2, AFG1,

AFG2), *A.flavus* only produces AFB1 and AFB2 (Calvo and Cary, 2015). The aflatoxin cluster, which contains aflatoxin biosynthesis genes as well as pathway-specific regulatory genes, consists of 25 genes including a 70 kb DNA section; this cluster is localized near the telomere of chromosome three (Amaike and Keller, 2014).

Cluster number	Location	Backbone enzyme	Decorating genes	LaeA regulation[a]	SM
1	IV R	PKS	9	yes	
2	IV R	DMAT	8	no	
3	IV R	NRPS	1	NA	
4	IV R	NRPS	4	yes	
5	IV R	PKS	3	yes	
6	VIII L	NRPS	12	yes	
7	VIII L	NRPS-like, PKS-like	10	yes	
8	VIII L	2NRPSs, PKS	9	no	
9	VIII L	2NRPSs, siderophore	10	no	
10	IV L	Arp1 conidial pigment	3	no	pigment
11	II R	NRPS-like	11	yes	
12	II R	NRPS-like	3	yes	
13	VII L	NRPS	4	no	
14	VII L	IroE-like siderophore	2	no	
15	VII L	DMAT	12	yes	aflatrem
16	VII L	sidA/sidR, siderophore	1	no	
17	I L	PKS, 2PKS-like	9	no	
18	I L	NRPS-like	6	no	
19	V L	DMAT	6	yes	
20	V L	2PKS	10	no	
21	VI R	2NRPS, ETP-like	36	yes	gliotoxin?
22	VI R	NRPS	6	yes	
23	VI R	PKS-NRPS hybrid, PKS	17	yes	
24	VI R	Pes1 NRPS	3	yes	
25	VI R	NRPS-like	8	yes	penicillin?
26	I R	PKS-like, 2NRPS-like	12	no	
27	I R	PKS	5	yes	
28	I R	NRPS-like	5	no	
29	I R	DMAT	2	no	
30	V R	DMAT, NRPS	6	yes	
31	V R	NRPS-like	14	yes	
32	V R	GGPP	19	yes	aflatrem
33	V R	2NRPS-like, PKS	8	no	
34	VI L	NRPS	8	no	
35	VI L	NRPS-like	5	yes	
36	III L	2PKS-like	7	no	
37	III L	NRPS-like	3	no	
38	III L	PKS	3	no	

Cluster number	Location	Backbone enzyme	Decorating genes	LaeA regulation[a]	SM
39	III L	PKS	5	no	
40	III L	PKS	9	no	
41	VII R	PKS	3	no	
42	VIII R	PKS	11	no	
43	VIII R	PKS-like, DMAT	18	yes	
44	VIII R	PKS	5	no	
45	VIII R	NRPS-like	16	yes	
46	VIII R	2PKS	4	no	
47	VIII R	NRPS-like	5	no	
48	VIII R	NRPS-like	19	yes	
49	II L	2PKS-like	6	no	
50	II L	PKS	7	yes	
51	II L	PKS	5	no	
52	II L	PKS	7	yes	
53	III R	NRPS	8	no	
54	III R	PKS	30	yes	aflatoxin
55	III R	DMAT, PKS-NRPS hybrid	4	yes	CPA

Fig.5 - Secondary metabolite clusters in *Aspergillus flavus.* The whole genome sequence in *A. flavus* identified 55 putative secondary metabolite clusters, based on backbone enzyme gene analysis by SMURF. Only a few numbers of clusters, such as aflatoxin, CPA, and aflatrem (two clusters), have been identified and characterized. Each backbone enzyme, such as polyketide synthases (PKS) and nonribosomal polypeptide synthases (NRPS), plays a major role in synthesizing/activating the clusters, and decorating enzymatic genes located beside backbone enzyme genes complete the synthesis (not shown in this table) completes synthesis (not shown in this table) (Amaike & Keller, 2011).

As we said before, there are 55 different clusters for secondary metabolism predicted by SMURF (a software - Secondary Metabolite Unknown Regions Finder- available at http://www.jcvi.org/smurf (Khaldi et al., 2010). Smurfit Institute of Genetics, Trinity College, Dublin, Ireland and Department of Infectious Disease, The J. Craig Venter Institute, Rockville, MD, USA) and they can be divided in four clades, deriving from the hierarchical clustering analysis:

- Clade A contains the Aflatoxin cluster (cluster 54) and eight other predicted gene clusters (1, 16, 19, 23, 24, 26, 36 and 55). Cluster 55 is necessary for the biosynthesis of CPA, cyclopiazonic acid;
- Clade B contains 11 gene clusters (4, 5, 9, 10, 11, 20, 23, 31, 37, 48 and 50);
- Clade C contains genes from 25 of the 55 predicted clusters and gene expression appeared to be low across almost all experimental conditions
- Clade D contains 18 different gene clusters slightly more expressed than those in clade C; it includes cluster 15, which is required for Aflatrem production.

Starting from the division in clades and the observation by Georgianna et al. (2010), where it been shown the connection between ecological conditions and fungal gene expression, Reverberi et al. (2013) examined genotypic and phenotypic changes in *A.flavus* during its growth and invasion of developing kernels. The fungus was grown under four different conditions that mimic ecological niches raging from saprophytic growth to parasitism but

A.flavus responded to growth conditions within a global transcription changes, resulting in differences in its ability to utilize carbon sources.

In those experiments, out of 56 secondary metabolites clusters known, 24 were differentially expressed; in a particular way, among the secondary metabolites that are up-regulated *in vivo*, the cluster 32 was the most interesting because within cluster 32 there are at least two genes involved in the synthesis of Aflatrem: Prenyl transferase AtmC and FAD-dependent monooxygenase AtmM (highlighted in yellow). For this reason we can therefore consider it as a cluster in cluster.

Affy Id	Description	Affy Id	Description
2541.m00053	Monooxygenase, putative	2541.m00042	TrkA-N domain dehydrogenase, putative
2541.m00052	Hypothetical protein	2541.m00041	Zn_2Cys_6 transcription factor
2541.m00051	Aromatic ring-opening dioxygenase family protein	2541.m00040	FAD dependent oxidoreductase, putative
2541.m00050	Aldehyde dehydrogenase PutA	2541.m00039	Prenyl transferase AtmC
2541.m00049	Conserved hypothetical protein	2541.m00038	FAD-dependent monooxygenase AtmM
2541.m00048	Salicylate hydroxylase, putative	2541.m00037	PTH11-like integral membrane protein, putative
2541.m00047	Acyl esterase	2541.m00036	Cytochrome P450, putative
2541.m00046	Cupin domain protein	2541.m00035	Acyl transferase, putative
2541.m00045	C_6 and C_2H_2 transcription factor	2541.m00034	NPP1 domain protein
2541.m00044	C_6 and C_2H_2 transcription factor	2541.m00033	Conserved hypothetical protein
2541.m00043	Hypothetical protein	2541.m00032	Conserved hypothetical protein

Fig. 6 - Description of *cluster 32*

The co-expression analysis applied by Mentenz et al. (2010) has been used for all secondary metabolic clusters to identify other genes co-regulated with these molecules; the results show that the cluster 32 should be essential, probably because it's co-expressed with Necrosis and Ethylene-inducing Peptide (*nepA*) which belongs to the NLPs (Nep-Like Proteins), a family of non-host specific elicitors causing necrosis and activating defense responses in dicotyledonous plants with a broad distribution across taxa (Keates et al., 2003; Staats et al., 2007)

These NLPs are small phytotoxic proteins conserved with the characteristic of inducing HR-like cell death, cell death, ROS and ethylene production. Recent structural analyses indicate that NLPs are similar to virulence promoting cytolytic toxins and function by interfering with integrity of the plasma membrane. The disruption of plasma membrane is detected by host plants leading to the activation of defenses (Laluk and Mengiste, 2010).

3. AFC-1

AFC-1 is an *Aspergillus flavus* strain derived by mutagenesis of wild type NRRL 3357. It is an Arginine and Uracil double nutritional auxotroph mutant, specifically engineered to obtain a strain of *A. flavus* with two selectable markers.

It has successfully been developed in Gary A. Payne's Laboratory (Raleigh, North Carolina) by Burroughs *et al*. in 2007.

The biosynthesis of Uracil and Arginine is critical to the survival of the fungus and these components must be supplied in growth media to strains that are incapable of synthetizing them.

In AFC-1, *pyrG* and *argD* genes are deleted: *pyrG* codifies for an Orotidine-5'-Decarboxylase and it's required for Uracil biosynthesis whereas *argD* codifies for an Acetyl Ornithine Aminotransferase and it's needed for Arginine biosynthesis.

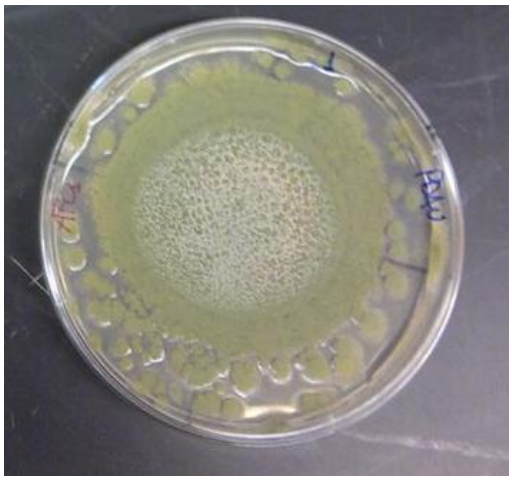


Fig. 7 - AFC-1 on PDAU

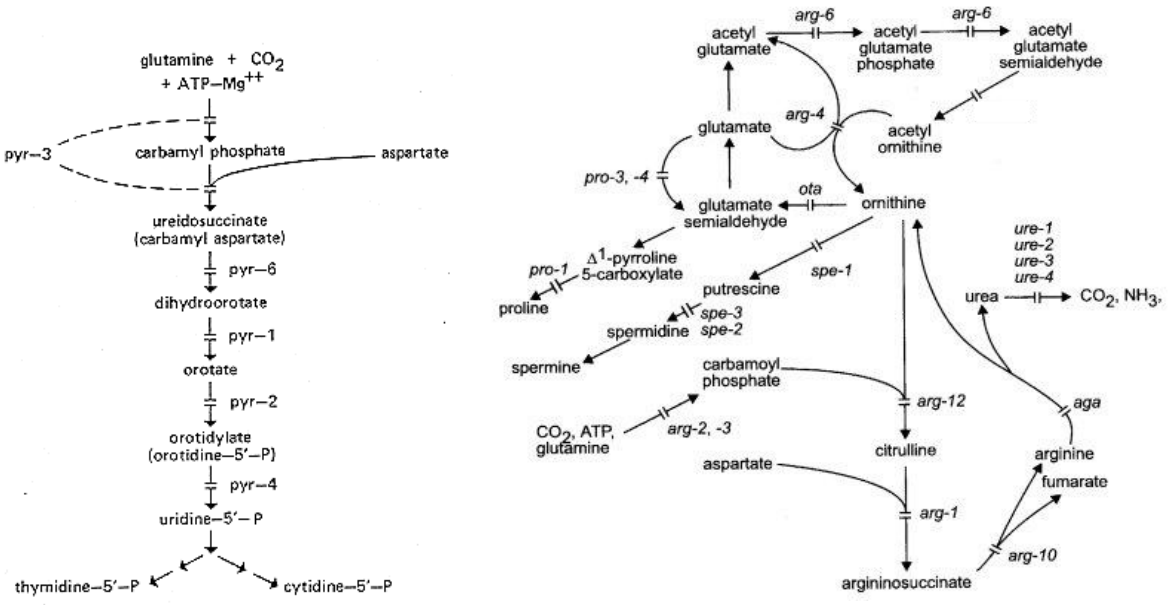


Fig. 8 - Uracil (on the left) and Ariginine (on the right) biosynthetic pathway

In order to create this double mutant, *A. flavus* strain 3357-5, which contains a nonfunctional copy of *pyrG*, was first utilized. Then an *argD* knock out construct was made. This construct was created with *pyr4*-blaster, a plasmid containing complementary Kanamycin cassette sequences flanking the *pyr4* gene from *Neurospora crassa* (the Kanamycin is not used for selection but only to facilitate removal of the *pyr4* selectable marker). On either side of these sequences, it was cloned in an upstream flanking region and downstream flanking region of *argD*. The construct was designed such that the marker gene can be forced to loop out under a selection marker.

Strain 3357-5 was then transformed with the knockout construct and transformants screened for the requirement of Arginine. The resulting Arginine auxotrophs were treated with 5-fluoro-orotic acid (5-FOA) which is converted to a lethal metabolite, fluorodeoxyuridine, by fungi that have a functional copy of an Orotidine-5'-Phosphate-Decarboxylase (*pyr4* or *pyrG*) (d'Enfert, 1996). By this method, it was possible to identify successfully colonies where the functional *pyr4* had been looped out. Then the double auxotroph screened was finally AFC-1.

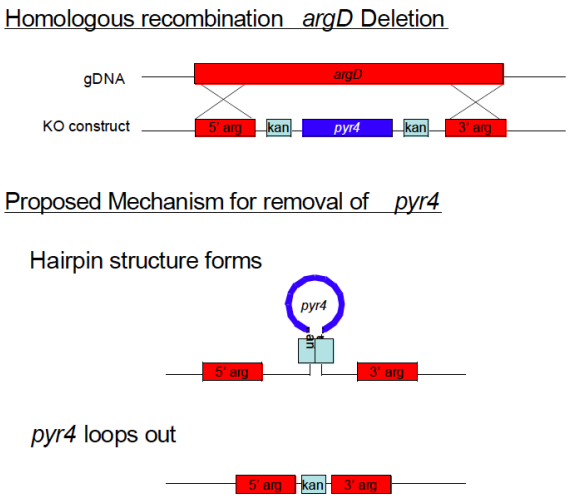


Fig. 9 - Mechanism for gene deletion and renewal of Uracil auxotrophy

Because of the economic and health significance of *A. flavus*, many efforts have been made to understand every aspect of its genetic regulation such as molecular control of Aflatoxin biosynthesis as well as the metabolic regulation of other secondary metabolites. Much of what is known today has been gathered through the manipulation of the *A. flavus* genome by means of a process called 'genetic transformation' which is to be considered a mean to incorporate foreign or modified DNA into an organism and can be successfully used to delete a gene or alter its expression. In filamentous fungi not all the cells within a population can be transformed at the end of the transformation protocol, therefore selectable markers are needed to differentiate between transformants and non-transformants.

Previous protocols proved that antibiotic resistance cannot be used successfully as selectable marker in *A. flavus* because of his naturally resistance to most common used antibiotics. For this reason nutritional auxotrophy has gradually become the primary mean of selecting transformants in filamentous fungi due to its simplicity.

4. PATHOGENICITY STRATEGIES OF HEMIBIOTROPHIC FUNGI

The lifestyle of plant pathogenic fungi are higly diverse and utilize distinct strategies to interact with host plants: necrotrofic fungi infect and kill host tissue and extract nutrients from dead host cells; biotrophic fungi colonize living host tissue and obtain nutrients from it; hemibiotrophic fungus show two phases during the infection process: the first one is an initial biotrophic phase followed by a necrotrophic stage. Particularly, successful colonization and infection of hemibiotrophic fungi depends upon the ability to modify host plants to take the necessary nutrients required for growth and reproduction (Selin et al.,

2016). The infection process of hemibiotrophic fungi require the devolpment of specialized structures as intracellular hiphae (Perfetc and Green, 2001).

Productions of virulence determinates referred to as “effectors” are the key governing factor that determinates host infection and colonization (Selin et al., 2016). These effectors are secreted in order to facilatate infection directly into the plant apoplast or cytolos; beside, most of understanding of effectors comes from studies of prokaryotic pathogens but less is know about effectors of filamentous pathogens, such fungi and oomycetes, but it’s clear that these pathogens secrete effectors to suppress defence responses or alter host metabolism. The first fungal effectors were identified in trying to clone Avr (avirulence) proteins recognized by host R (resistance) proteins. The fungal effectors contain canonical secretion signals and appaer to be secreted through the standard endomembrane pathway; some insight into the process of movement into plant tissue has come from finding that oomycete pathogenes (Koeck et al., 2011).

Biotrophic and hemobiotrophic fungal pathogens feed and live on living host cells, and secrete effectors that are targeted for the host apoplast or cytoplasm using specialized infection structures such as appressoria or haustoria. Hemibiotrophic pathogens utilize a biotrophic phase early in the infection process followed by a necrotrophic phase killing host cells to complete their lifecycle. For the hemibiotrophic pathogen *Magnaporthe oryzea*, two discrete secretion systems for delivery of apoplastic and cytoplasmic effectors are used. For cytoplasmic effectors delivery, these proteins appear to accumulate in the biotrophic interface complex (BIC) near the tip of the first bulbous cell formed after host cell penetration. Apoplastic effectors, on the other hand, are not associated with BIC, and once secreted they are dispersed in the extracellular space between the fungal cell wall and the extra-invasive-hyphal membrane. (Selin et al., 2016).

However, filamentous pathogen effectors are often highly diverse in sequence and structure and most likely evolved various mechanisms for delivery into the host (Scala et al 2016). Future studies are required on effectors functions during infection, including effector delivery and cell-to-cell movement in planta and, also, it remains unclear how fungal effectors target host immunity (Dong et al., 2015).

5. *ZEA MAYS*

Maize (*Zea mays* L.) was domesticated from its wild relative, the teosinte *Zea mays* ssp. *parviglumis* Iltis & Doebley, in the south-western Mexico during the early Holocene (Bracco et al., 2016).

Fig. 10 - Maize Cobs

The first genetic linkage map of maize was constructed in 1986 based on restriction fragment (RFLP) in the F_2 mapping population of a cross between H427 and 761. Subsequently, with new molecular techniques, it will be generate a highly saturate genetic linkage map (Zhou et al., 2016). From the genome map, there's some gene duplications exist in maize: the additional copies of genes can introduce functional redundancy, which may promote evolutionary processes at either the coding or regulatory level, as example the resistance against pathogens (Li et al., 2016). Maize is one of the most economically important and widely grown crops are used for human food, livestock feed and alcohol (Shu et al., 2015). Molecular breeding is a promising method to bolster maize productivity and the majority of agriculturally important traits in maize are quantitative and controlled by numerous genes. Plant height is related to lodging resistance and harvest index during crop production (Wang et al., 2016). Because of its importance, researches are concerning the problem of disease since plant pathogens, like *Aspergillus flavus*, and the presence of mycotoxins on crops. Normally maize contains low levels of aflatoxins, but in the last years contamination levels frequently exceed the levels permitted for commerce and remediation by diluting the contaminated meal with less contaminated grain or by chemical treatment to destroy or inactivate aflatoxins is necessary (Ehrlich, 2014).

Furthermore, maize kernels are generally susceptible to infection by *A.flavus* but during the study of this process, it's been found almost 16 genes highly expressed in the resistant variety of maize involved in resistance to aflatoxin accumulation and 15 genes in the susceptible variety. These observations led to the conclusions that multiple mechanism are likely involved in resistance to aflatoxin contamination (Dolezal et al., 2014).

6. AIM OF THE THESIS

Filamentous fungi are one of the most important subject of study for plant pathologists' and molecular biologists'. Their incredible adaptability to diverse environmental conditions, their ubiquitary diffusion and the ability to produce an enormous variety of secondary metabolites, some of which carcinogenic, have always fascinated as well as worried scientists worldwide.

Aspergillus flavus is a widespread distributed filamentous fungus that produces some secondary metabolites among which aflatoxins that are extremely cancerogenic, mutagenic, teratogenic mycotoxins associated with acute and chronic toxicity in humans and animals (Wang et al. 2016).

In this study, we resolved the aim of adding even a small but important piece of the complex framework that outlines the mechanisms by which *Aspergillus flavus* interacts so dynamically with environment, particularly with his host *Zea mays*.

Relying on the excellent results achieved by pathologists in the last 10 years and thanks to the refinement of the techniques of molecular investigation, we focused on *A. flavus* - maize kernels interaction, paying specific attention to some of the molecular players involved in the process: for this reason, considering relevant the role of Cluster *32* in pathogenesis, we studied *AFLA_096370* gene which encodes for a sequence-specific DNA-binding binuclear Zinc protein (Zn_2Cys_6), one of his transcription factor, outlining its part in gene expression.

Fig. 11 - Zn_2Cys_6 transcription factor (AFLA_096370)

Zn_2Cys_6 is required for transcriptional activation of many structural genes and it belongs to a protein cluster unique to Fungi that have been reported to be involved in different regulatory functions like fungal growth, sexual and asexual development, conidial germination, appressorium formation, pathogenicity and response to stress (Zhao *et al.*, 2011; Lu *et al.*, 2014) and production of secondary metabolites and asexual and sexual development (Chang and Ehrlich, 2013).

al., 2011; Lu *et al*., 2014).

The canonical members of this class contain a binuclear Zinc domain in which two Zinc ions are bounded by six Cysteine residues forming an N-terminal Zn_2Cys_6 binuclear cluster whereas C-terminal is helical and takes part to a coiled-coil dimerization element; a nine-

residue extended strand termed ‘linker’ connects the amino- and carboxyl-terminal domains (Liang *et al*., 1996). Zn_2Cys_6 transcription factor binds as a homodimer to DNA targets containing two inverted CGG half-sites.

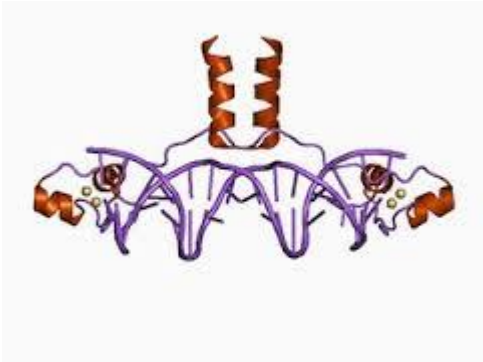


Fig. 12 - Zn_2Cys_6 schematic representation

Because of great challenges and workload in deleting genes on a large scale, the function of most of these secondary metabolic genes are still unclear.
Since the function of most of secondary metabolites genes is unknown, in this study we used different constructs.

We developed:
1) A high-throughput gene knock-out system *via* the TOPO cloning method in order to obtain a Zn_2Cys_6 knock-out mutant; we used the deletion construct to transform AFC-1, a double auxotroph mutant incapable of producing Arginine and Uracil (the screening was performed through PCR assay). Once obtained, simultaneously, to better characterize the metabolic profile related to Cluster *32*, we produced overexpression mutants of Zn_2Cys_6 fused to GFP (the screening was performed basing on fluorescence emission).
Such mutants have finally been tested to assay pathogenicity and fitness in different environmental conditions, compared to the wild type with regard to expression profile of some pathogenesis-related genes.
2) A overlap fusion PCR with pyrithiamine antibiotic resistance to transform *A.flavus* NLRR 3357 (the screening was performed through PCR assay).
At the same time, to understand the first steps of infection cycle we have taken two ways: the first one compare with an histological assay on fungal strains grown on *Zea mays*; the second one, we exploit the GUS assay for two mutants of the necrotrophic factor, nepA (one is a knock-out and one is a over-expressing), on *Zea mays* as substrate.

In order to studying *A.flavus* life cycle and infection process, we have investigated the role of two hormones, key of defense responses of host plant, *Zea mays*, in this case, salicylic acid and jasmonic acid, using HPLC-MS/MS. The first one, the salicylic acid, is often considered to trigger defenses effective against biotrophic pathogens and the jasmonic acid, besides, triggers defenses usually more effective against necrotrophic pathogens.

MATERIALS AND METHODS

1. STRAINS OF *ASPERGILLUS FLAVUS*

- WT strain (wild-type) Af3357, kindly provided by the laboratory of Professor G.A. Payne (CIFR, North Carolina State University, Raleigh, U.S.A.);
- AFC-1 strain, double auxotroph for Arginine and Uracil, kindly provided by the laboratory of Professor G.A. Payne (CIFR, North Carolina State University, Raleigh, U.S.A.);
- A1 ad A2 mutants: auxotrophs for uracil; they derive from AFC1 strain in which the arginine auxotrophy has been restored;
- C32 OE mutant: auxotroph for uracil; it derives from A1 mutant for subsequent re-insertion of the transcription factor under an over-expression promoter;
- NepA OE-GUS B5-12 strain: nepA over-expressing strain, provided by X. Shu under the direction of Professor G.A. Payne (CIFR, North Carolina State University, Raleigh, U.S.A.);
- NepA KO-GUS B9-5 strain: nepA knock-out strain, provided by X. Shu under the direction of Professor G.A. Payne (CIFR, North Carolina State University, Raleigh, U.S.A.).

2. BACTERIAL STRAINS

For the cloning of genomic fragments of interest, One Shot® competent *E. coli* cells, stored at -80 °C, were used (TOPO® TA Cloning® Kit, Invitrogen).

3. PLASMIDS

For the realization of two constructs, the knock out and the over-expression one, two different plasmids were used:

- pCR 2.1 TOPO vector (3931 bp), Invitrogen;
- pNUC'EM2 (6192 bp) designed by D. Kerr and K. Wells (Science Institute, Gene Evaluation and Mapping Laboratory, Beltsville, Maryland 20705) and kindly provided by the laboratory of Professor G.A. Payne (CIFR, North Carolina State University, Raleigh, U.S.A.);
- HM640258.1 (7664 bp) kindly provided by Dr. Jeff W. Cary, SRRC, New Orleans, USDA.

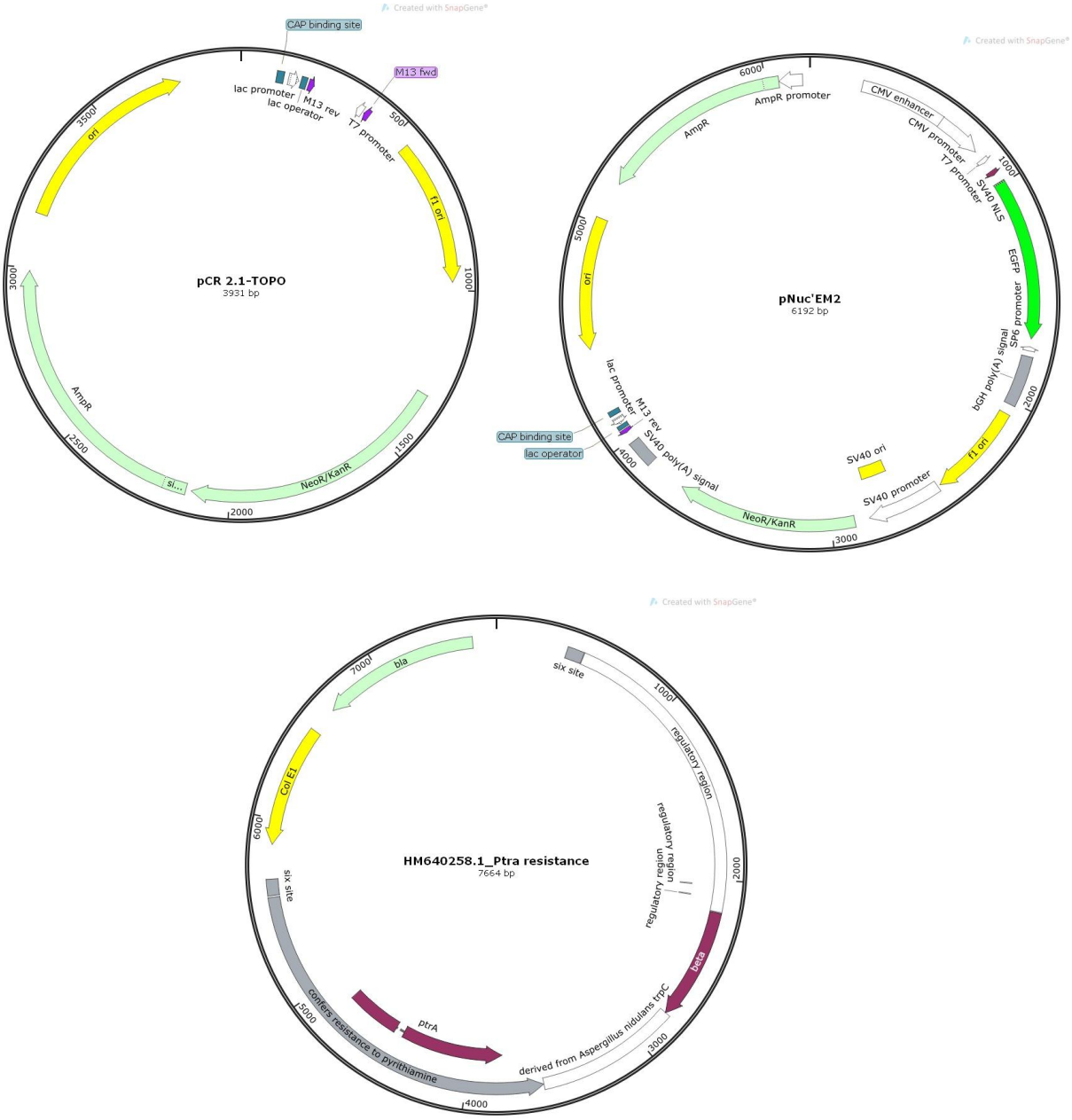


Fig. 13 - Plasmids for protoplasts transformation: pCR 2.1 TOPO vector, pNUC'EM2, HM640258.1(Ptra resistance)

4. *ZEA MAYS* CROP

The maize used is Pioneer P1543 classe FAO 600 from 130 days, gently given by "Agricola 200"

The maize is prepared after two washes with autoclaved water plus bleach 60% for 20 minutes and then it was washed with autoclaved water.

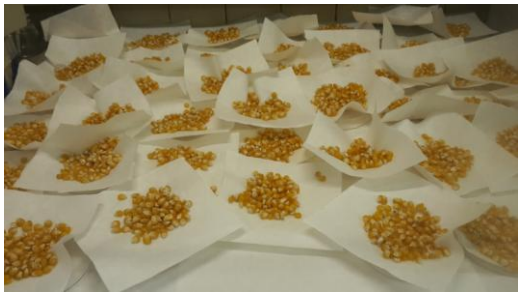


Fig. 14 - *Zea Mays*

5. CULTURE MEDIA

- Czapek Dox Broth or Agar (CD, Difco)
- Czapek Dox Broth or Agar (CD, Difco) with Uracil 1.122 g/L
- Czapek Dox Broth or Agar (CD, Difco) with Uracil 1.122 g/L and 0.26 g/L of Arginine
- MLS: Czapek Dox Broth or Agar (CD, Difco) with 52.86 g/L of Ammonium Sulphate (NH_4SO_4, 0.4M)
- MLS+U: Czapek Dox Broth or Agar (CD, Difco) with Uracil 1.122 g/L and 52.86 g/L of Ammonium Sulphate (NH_4SO_4, 0.4M)
- Potato Dextrose Agar (PDA, Difco)
- Potato Dextrose Agar (PDA, Difco) with Uracil 1.122 g/L
- Potato Dextrose Broth (PDB, Difco) with Uracil 1.122 g/L
- CZU: Czapeck Dox Agar (CD, Difco) with Ammonium Sulphate (NH_4SO_4, 0,5 M) and (KCL, 3M)
- Luria Bertani Broth or Agar (LB, Difco)
- S.O.C. (Bacto-tryptone 2%, yeast extract 0.5%; NaCl 10 mM; KCl 25 mM; Mg2 + 10 mM glucose and 20 mM)

All substrates were sterilized by autoclave at 121°C for 20 minutes.

6. C-TAB METHOD FOR FUNGAL DNA ISOLATION OF *A. FLAVUS* (STARTING FROM MYCELIUM)

30 mg of mycelia freeze-dried and pulverized in liquid nitrogen + 500 µL of buffer C-TAB I (previously heated to 65 ° C), 500 µL of solution 2A and 5 µL of proteinase K (20 mg/mL); o.n. at 55°C in gentle stirring.

The day after, 20' at 65°C, 10' on ice for 10 minutes.

Spin 15' at 4C at 13000 rpm.

Add 3/10 V Sodium Acetate at the recovered supernatant, incubate 30' on ice.

Spin 10' at 4C at 13000 rpm.

Add 1 volume (v/v) of Phenol:Chloroform:Isoamyl Alcohol (25:24:2) at supernatant .

Spin 10 min. at 4C at 13000rpm.

Add 1/10 (of the volume) of C-TAB II and 1 volume of Chloroform:Isoamyl Alcohol (24:1) to the recovered surnatant.

Spin 5 min. at 4C at 13000rpm.

Add 1 volume of C-TAB III and 1 volume of Chloroform:Isoamyl Alcohol (24:1) to the recovered surnatant. gently emulsify by inversion, incubate at R.T. for 30 min.

Spin 5 min. at 4C at 13000rpm.

Add 0.6 volumes of cold isopropanol to the recovered surnatant

Spin 30 min. at 4C at 13000rpm.

Wash DNA pellet with 70% ETOH

Air dry (or in a stove at 56°C) pellet 30 min and resuspend in 50uL of sterile water and 2 µL of RNase (20 mg/mL).

Solutions

C-TAB I C-TAB 4%	C-TAB II C-TAB 10% NaCl 0.7M
C-TAB III C-TAB 1% Tris-HCl 50 mM pH8 EDTA 10 mM	2A Solution NaCl 2,8M Tris-HCl 200 mM pH8 EDTA 40 mM

7. C-TAB METHOD FOR FUNGAL DNA ISOLATION OF *A.FLAVUS* (STARTING FROM SPORES)

Add 50 µL of spores collected by Triton-X100 to 500 µL of C-TAB Buffer and one scoop of glass beads.

Vortex using Disruptor Gene 2' and incubate at 65°C for 15 minutes. Repeat this step twice.

Add 500 µL of Chloroform:Isoamyl alcohol (24:1) mix and spin 5' at 13000 rpm.

At the recovered supernatant add 233 µL of Isopropanol and 32 µL of Ammonium Acetate 7.5M; Mix and spin 15' at 13000 rpm.

Supernatant is poured off and pellet is washed with 500 µl of cold 70% Ethanol.

Spin 5' at 13000 rpm.

Air dry (or in a stove at 56°C) pellet 30 min and resuspend in 50uL of sterile water and 2 µL of RNase (20 mg/mL).

This protocol has been kindly provided by Gary Payne's Lab, North Carolina State University, NC-USA.

Solutions

C-TAB stock, 1lt		C-TAB BUFFER	
100 mL	Tris 1M pH8	5 mL	C-TAB stock
280 mL	NaCl 5M	0.2 g	PolyVinylPyrrolidone
40 mL	EDTA 0.5M	25 µL	β-mercaptoethanol
20 g	CTAB		
Water	bring to volume and Autoclave		

8. RNA EXTRACTION AND QUANTIFICATION

Add 1 mL of cold Trizol (Invitrogen, Carlsbad, CA, USA) to 50 mg of lyophilized and grinded matrix.

Mix and spin 5' at 1000 rpm at 4°C.

Add with 200 µl of Chloroform at the supernatant, mix and spin.

Add 1 volume of Chloroform: Isoamylic alcohol, mix and spin.

Add 500 µl of cold iPrOH and spin.

Discard the upper phase and wash pellet with cold Ethanol:DEPC water 70 % v/v.

Dry pellet and resuspend with sterile DEPC water, heat-shock at 70 °C and store at -80°C.

RNA was quantified trough Qubit RNA Assay kit.

Pellet formation was increased trough another centrifuge step. The upper phase was then discarded and then the pellet was washed with The pellet was oven dried and suspended in a solution, then.

9. PRODUCTION OF KNOCK-OUT CONSTRUCT

9.1 Knock-out system *via* Fusion PCR

Following Szewczyk et al. (2007), the first PCRs was made to amplify the upstream and downstream region of the pyrithiamine cassette and pyrithiamine itself. The second and third amplification were made using 'nested' primers to merge the fragments into a single molecule (5784 bp).

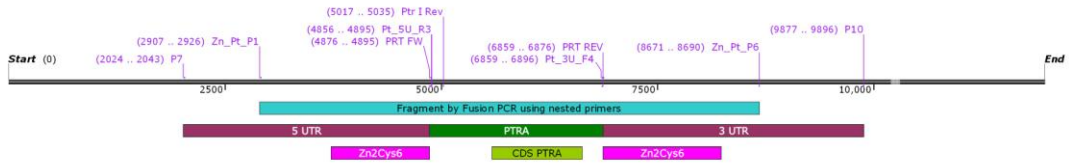


Fig. 15 - Fusion PCR fragment, with highlighted primers used to the amplification (using Snapgene software)

Primers for Pyrithiamine gene:

Ptr Fw	GGGCAATTGATTACGGGATC	2001 bp
Ptr Rev	TGACGATGAGCCGCTCTT	

PCR conditions:

Ex Taq (TaKaRa) 10X Buffer	5 µL
dNTPs Mix (TaKaRa) 2.5 mM each	2 µl
Control PCR Primers (10 µM)	0,5 µL each
Control DNA (plasmid)	1 ng
Taq Polymerase (Ex TaKaRa)	1,25 U
Water	to volume
V_f	50 µL

PCR settings:

Step	Time	Temperature	Cycles
Initial denaturation	4'	94°C	1X
Denaturation	30"	94°C	25X
Annealing	20"	60°C	
Extension	2'	72°C	
Final extension	5'	72°C	1X
Hold		4°C	

Primers used to amplify the flanking regions of Zn_2Cys_6 (The green section indicates the overlap section between the flanking regions and the pyrithiamine):

Zn_Pt_P7	gatatggtgtgctccgagga	2872 bp (5 UTR)
Pt_5U_R3	GATCCCGTAATCAATTGCCCcgctgcttacgagtcaggtt	

Pt_3U_F4	AAGAGCGGCTCATCGTCAccgagacgctccttctactg	3038 bp (3UTR)
Zn_Pt_P10	ccattcgtccaccattgcat	

PCR conditions:

Ex Taq (TaKaRa) 10X Buffer	5 µL
dNTPs Mix (TaKaRa) 2.5 mM each	2 µl
Control PCR Primers (10 µM)	0,5 µL each
Control DNA	100 ng
Taq Polymerase (Ex TaKaRa)	1,25 U
Water	to volume
V_f	50 µL

PCR settings:

Step	Time	Temperature	Cycles
Initial denaturation	4’	94°C	1X
Denaturation	30”	94°C	26X
Annealing	20”	60-65°C	
Extension	3’	72°C	
Final extension	5’	72°C	1X
Hold		4°C	

T_{ann}= 65°C for 5UTR and 60° for 3 UTR

The PCR products (Results: Fig. 21) have been purified on columns (Isolate Isolate II PCR and Gel Kit - Bioline) and cecked on agarose gel 1%.

“Nested” primers used for fusion PCR:

Zn_Pt_P1	cccatccatactaccacccc	5784 bp
Zn_Pt_P6	cgcaaggcaaggattcgtta	

In order to obtain the fusion product a first step was taken to bind the 5UTR fragment to the pyritiamine, using one of the two nested primers (Zn_Pt_P1) and a special Taq Polymerase (TransStart® FastPfu DNA Polymerase -Transgenbiotech)

PCR conditions:

5×TransStart® FastPfu Buffer	10 µL
dNTPs Mix (2.5 mM) each	4 µL
PCR Primers (10 µM) Zn_Pt_P1 / Ptr Rev	1 µL each
DNA (purifiedon column)	30 ng 5'UTR 20 ng PTRA
TransStart® FastPfu DNA Polymerase 2.5U/µl	2,5 U
Water	to volume
V_f	50 µL

PCR settings:

Step	Time	Temperature	Cycles
Initial denaturation	2’	95°C	1X
Denaturation	20”	95°C	30X
Annealing	20”	58°-60°C	
Extension	2’	72°C	
Final extension	5’	72°C	1X
Hold		4°C	

The PCR products(3970 bp) have been purified on columns (Isolate Isolate II PCR and Gel Kit - Bioline) and cecked on agarose gel 1% (Results: Fig. 22).

In the last step the product obtained from the union of the first two fragments was merged to 3 UTR, through nested primers.

PCR conditions:

5×TransStart® FastPfu Buffer	10 µL
dNTPs Mix (2.5 mM) each	4 µL
$MgSO_4$ 50 mM	1 µl
PCR Primers (10 µM) Zn_Pt_P1 / Zn_Pt_P6	1 µL each
DNA (purifiedon column)	40 ng 5'UTR+PTRA 20 ng 3UTR
TransStart® FastPfu DNA Polymerase 2.5U/µl	2,5 U
Water	to volume
V_f	50 µL

PCR settings:

Step	Time	Temperature	Cycles
Initial denaturation	2’	95°C	1X
Denaturation	20”	95°C	30X
Annealing	20”	58°-60°C	
Extension	3’	72°C	
Final extension	5’	72°C	1X
Hold		4°C	

The PCR products have been purified on columns (Isolate Isolate II PCR and Gel Kit - Bioline) and cecked on agarose gel 1% (Results: Fig. 23).

As the PCR product showed nonspecific amplifications, it was purified from gel and precipitated with Sodium Acetate to be able to use it for the subsequent transformation of protoplasts.

DNA precipitation with Sodium Acetate and Ethanol

- Measure volume of DNA solution (max 300 µl). Then add 0,1 V of 3 M Na-Acetate pH 5,2 and 2,5 V 100% cold EtOH
- After adding the NaAc and EtOH, cap, mix briefly, and incubate 1 hour at -20°C
- Centrifuge on high (13000 rpm) at 4°C
- Carefully pour out the supernatant
- Wash the pellet by adding 1 ml 70 % EtOH
- Discard super as before
- Remove last traces of 70 % EtOH using a drawn out glass pipette or equalivent
- Dry in a stove at 56 ° C for 20'
- Ready for resuspension (max 10 µl)
- Pellet obtained is re-suspended in 10 µL of sterile water

9.2 Knock-out system *via* the TOPO cloning for Zn_2Cys_6

The assembly of the three fragments (5'UTR and 3'UTR of Zn_2Cys_6 and *arg*D) has been reached through the use of 4 restriction enzymes belonging to the family of Eight-Base Cutters. Their specific restriction sequences have been added to the primers designed with PRIMER3 INPUT software (Tab. 1).

*Asc*I, *Fse*I, *Sbf*I-HF, *Pac*I and the Cut Smart Buffer have been provided by New England Biolabs (NEB).

5U1	GGCGCGCCTCAGTGAGGCTCGTGGTTTT	AscI	5'...G G↓C G C G C C...3' 3'...C C G C G C↑G G...5'	5'UTR 1558 bp
5U2	GGCCGGCCAGATTTACGCCTTGGCTCCT	FseI	5'...G G C C G G↓C C...3' 3'...C C↑G G C C G G...5'	
ArgFw	GGCGCGCCttaaatGGCCGGCCaattgcggagcaaatcaca	AscI-ttaaat-FseI		*arg*D 2356 bp
ArgRev	TTAATTAA gccgggCCTGCAGGttcctctgatgcagggattc	PacI-gccggg-SbfI-HF		
3U3	CCTGCAGGGACTAAGCCTCGCCCATTTG	SbfI-HF	5'...C C T G C A↓G G...3' 3'...G G↑A C G T C C ...5'	3'UTR 1491
3U4	TTAATTAATTGATCAGGCAAGGTCCTCG	PacI	5'...T T A A T↓T A A ...3' 3'...A A T↑T A A T T...5'	

Tab. 1 - Primers specifically designed to amplify the individual fragments and containing the restriction sites of the eight-base cutters used

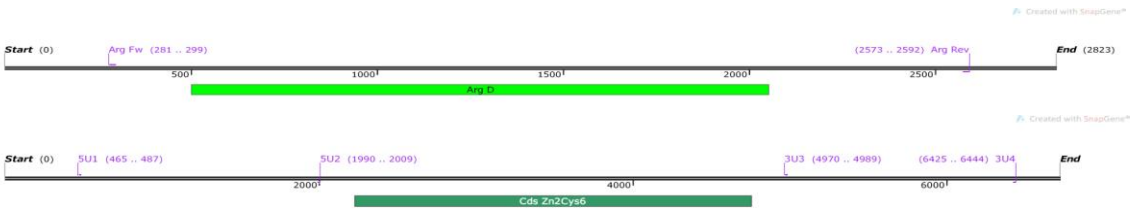


Fig. 16 - *argD* (up) and Zn_2Cys_6 (below) sequences (showing primers specifically designed to amplify the individual fragments) obtained through the use of Snapgene software

PCR conditions

10X PCR Buffer (Biotaq Bioline)	5 µL
$MgCl_2$ (50 mM)	2 µL
dNTP Mix (2,5 mM each)	1 µL
Control PCR Primers (10 µM)	1 µL each
Control DNA Template (100 ng/uL)	1 µL
Taq Polymerase (Biotaq Bioline) (5 unit/µL)	0,25 µL
Water	bring to volume
V_f	50µl

PCR settings for amplification of 5'UTR, 3'UTR (on the left) and *argD* (on the right). (For gel photo see Results Fig. 26)

Step	Temperature	Time	Cycles
Initial denaturation	96°C	4'	1X
Denaturation	96°C	15"	30X
Annealing	55°C	15"	
Extension	72°C	2'	
Final extension	72°C	5'	1X
Hold	4°C		

Step	Temperature	Time	Cycles
Initial denaturation	96°C	4'	1X
Denaturation	96°C	15"	40X
Annealing	55°C	15"	
Extension	72°C	2'15"	
Final extension	72°C	5'	1X
Hold	4°C		

Each fragment is individually cloned into plasmid TOPO vector pCR 2.1 (3931 bp) with TOPO® CLONING KIT (Invitrogen) and used to transform One Shot® TOP10 chemically competent E. coli (according to the procedure reccomended by kit).
Selected colonies (*via* blue/white screening) are analyzed by plasmid isolation and PCR (Fig. 28 Results)

Plasmids containing fragments are extracted using MINI PREP KIT (Sigma) and checked using PCR (so as to control the actual insertion of the fragment)(Results: Fig. 29).
After this step, plasmids containing *argD* and the ones containing 5'UTR are digested

(separately) o.n. at 37°C with AscI and FseI enzymes, starting with the assembly of the construct. During this double digestion, the entire 5'UTR fragment is excised while the plasmid containing *argD* is simply linearized (Results: Fig. 30).

Double Digestion pCR 2.1 + *argD* /pCR 2.1 + 5'UTR (V_f=50µL)

10x Cut Smart Buffer	5 µL
DNA	1-2 µg
AscI	1µL
FseI	1µL
H_2O	to volume
V_f	50 µL
Incubate o.n. at 37°C	

After this step, 5'UTR and TOPO vector are separated by gel electrophoresis: bands of interest are then cut and purified with Sigma Kit.
After purification and check of DNA by electrophoresis, a quantification (using Nanodrop) is necessary in order to prepare the forward step of Ligation.

Ligation of pCR 2.1 *argD* / 5'UTR

T4 DNA Ligase Buffer 2X	10 µL
pCR 2.1 + *argD*	200 ng
5'UTR	120 ng
T4 DNA Ligase	1 µL
H_2O	to volume
V_f	20µL
Incubate o.n. at RT	

In order to screen for plasmids in which the effective ligation has occurred, transformation of TOP10 cells is necessary, cells are grown on LBA+Amp100. Amongst CFUs which have grown, 10 colonies have been selected and plasmids are recovered using MINI PREP KIT (Sigma).
At the end of this Ligation step, a PCR control is necessary to be sure of 5'UTR insertion into the plasmid containing a*rgD* (Results: Fig. 31).

PCR Conditions:

10X PCR Buffer (Biotaq Bioline)	5 µL
$MgCl_2$ (50 mM)	2 µL
dNTP Mix (2,5 mM each)	1 µL
Control PCR Primers (10 µM)	1 µL each
Control DNA Template	100 ng
Taq Polymerase (Biotaq Bioline)	1,25 U
DMSO (4% finale)	2 µl
Water	to volume
V_f	50 µL

PCR settings:

Step	Time	Temperature	Cycles
Initial denaturation	1’	94°C	1X
Denaturation	10”	94°C	26X
Annealing	30”	68°C	
Extension	4’	72°C	
Final extension	9’	72°C	1X
Hold		4°C	

This first passage is followed by a second double digestion of the previusly obtained plasmid (5’UTR + *argD*) and of the one in which 3’UTR was cloned, using restriction enzymes SbfI-HF and PacI; through a gel electrophoresis, bands of interest are separated, cutted out and purified using GenElute Gel Extraction Kit (Sigma) (Results: Fig. 32).
In the last step pCR 2.1 plasmid containing 5’UTR + *argD* is incubated together with linear 3’UTR to finally obtain the whole construct (Results: Fig. 34).

LIGATION OF pCR 2.1 + 5'UTR + *argD* / 3’UTR

T4 DNA Ligase Buffer 2X	10 µL
pCR 2.1 + 5’UTR + *argD*	200 ng
3’UTR	120 ng
T4 DNA Ligase	1 µL
H_2O	to volume
V_f	20µL
Incubate o.n. at RT	

The pCR 2.1 plasmid is now used to transform TOP10 cells, which are selected on LBA+Amp100 medium. 6 CFUs are identified and grown on liquid LB; plasmids are recovered using Mini Prep Kit (Sigma). The digestion of knockout construct with EcoRI and successive control PCR of the six colonies selected as positive, confirmed results obtained (Results: Fig. 33).

PCR Conditions

10X PCR Buffer (Biotaq Bioline)	5 µL
$MgCl_2$ (50 mM)	2 µL
dNTP Mix (2,5 mM each)	1 µL
Control PCR Primers (10 µM)	1 µL each
Control DNA Template	100 ng
Taq Polymerase (Biotaq Bioline)	1,25 U
DMSO (5% finale)	2,5 µl
Water	to volume
V_f	50 µL

PCR Setting

Step	Time	Temperature	Cycles
Initial denaturation	1’	94°C	1X
Denaturation	10”	94°C	25X
Annealing	30”	68°C	
Extension	6’	72°C	
Final extension	9’	72°C	1X
Hold		4°C	

10. PRODUCTION OF OVER-EXPRESSION CONSTRUCT

Using PRIMER 3 INPUT Software, primers *ad hoc* are designed adding restriction sites of *Hind*III to the Forward end and *Kpn*I to the Reverse for Zn_2Cys_6 amplification.

ZnFW1Hind	AAGCTTgggccgaatcttaatcgtcg
ZnR2Kpn	GGTACCacatgaacactgggtagcga

With these primers and using a PCR, Zn_2Cys_6 is amplified and subsequently purified (GenElute™ PCR Clean-Up, Sigma) (Results: Fig. 35).

PCR Conditions:

10X PCR Buffer (Biotaq Bioline)	5 µL
$MgCl_2$ (50 mM)	2 µL
dNTP Mix (2,5 mM each)	1 µL
Control PCR Primers (10 µM)	1 µL each
DNA	100 ng
Taq Polymerase (Biotaq Bioline)	1,25 U
Water	to volume
V_f	50 µL

PCR Settings:

Step	Time	Temperature	Cycles
Initial denaturation	4’	96°C	1X
Denaturation	30”	96°C	30X
Annealing	15”	61°C	
Extension	3’	72°C	
Final extension	10’	72°C	1X
Hold		4°C	

After Zn_2Cys_6 purification, the DNA is quantified.

Respecting the equimolar *ratio* 1: 3 (a mole of vector: three moles of insert), we proceed with the Ligation of gene of interest in pCR 2.1 TOPO vector :

LIGATION of pCR 2.1. TOPO vector with Zn_2Cys_6

pCR 2.1 vector	10 ng
insert (Zn_2Cys_6)	25 ng
Salt Solution	1µL
H_2O	to volume
V_f	6µL
Incubate 30' at room temperature. Centrifuge the ligation reactions briefly and place on ice	

Select colonies (*via* blue/white screening) and analyze by plasmid isolation and PCR (see upper conditions and Settings; primers ZnFW1Hind/ ZnR2Kpn).

The next step strategy expects the excision of Zn_2Cys_6 gene now cloned into the pCR 2.1 vector and the following cloning of the latter in pNuc'Em2. All this is achieved by designing two double digestions, respectively of pCR 2.1 vector + Zn_2Cys_6 and of pNuc'Em2, with *Hind*III and *Kpn*I enzymes.

Double Digestion pCR 2.1 vector + Zn_2Cys_6 / pNuc'Em2

10x Cut Smart Buffer	5 µL
DNA	1-2 µg/µL
HindIII	1 µL
KpnI	1 µL
H_2O	to volume
V_f	50µL
Incubate at 37°C for 1,5h	

At the end of the digestion process, pNuc'Em2 is linearized while Zn_2Cys_6 is totally excided from pCR 2.1 vector (Results: Fig. 36).

After double digestion, through a gel electrophoresis, bands of interest (Zn_2Cys_6 and pNuc'Em2 vector) are cut out and purified using purification kit Sigma (GenElute™ Gel Extraction Kit, Sigma) (Results: Fig.37).

After quantification of DNA, Ligation is the forward step: it occurs through incubation of pNuc'Em2 vector with Zn_2Cys_6 gene, following the equimolar *ratio* 1: 3.

Ligation pNuc'Em2/ Zn_2Cys_6 (V_f=10 µL)

Buffer 2X (Promega)	5 µL
pNuc'Em2	100 ng
Insert (Zn_2Cys_6)	160 ng
DNA T4 Ligase	1 µL
H_2O	to volume
V_f	10 µL
Incubate o.n. at RT	

When Zn_2Cys_6 is cloned into pNuc'Em2, this plasmid is used for Transformation of competent TOP10 cells (as required by protocol previously mentioned). Plasmid pNuc'Em2+Zn_2Cys_6 is recovered with GenElute ™ Plasmid Miniprep Kit, Sigma. Using primers for amplification of Zn_2Cys_6, selected colonies (10) are later tested for the presence of the gene of interest by using a simple PCR program (see upper conditions and Settings; primers ZnFW1Hind/ ZnR2Kpn) (Results: Fig.38).

Given the size of the fragment (2771 bp), very large compared to cloning capacity of the vector, these experiments were repeated several times (Results: Fig.39)

11. TRANSFORMATION OF *A.FLAVUS* NRRL 3357 AND AFC-1

First, an overnight culture of wild-type NRRL 3357 has to be started by putting 5-6 mL of distilled and sterile water and Triton X-100 (0,01%, Sigma) on a sporulating plate (5-10 days old) and scraping the whole plate using a glass spreader bar; spores, collected in this way, are inoculated into 100 mL of PDB and grow at 28°C for 12-13 hours in agitation (200rpm); the same growth culture of AFC-1 was started with the same conditions of wild-type but using 100 mL of PDB+U+A.

When spore germination occurred, the overnight culture is poured into 50 mL falcons (100 mL cultures divided into two falcons) and centrifuged for 10' at around 8000rpm in a swinging bucket. Supernatant is then poured off while the pellet is re-suspended with sterile water and centrifuged at 4000rpm for 12'. After the centrifugation, supernatant is discarded and the solution containing the enzymes of digestion, appropriately filter-sterilized, is added. The enzyme solution (15 mL for each falcon) has to be dispensed dropwise (at the end of the protocol).

At this point, the mycelium, to which the lytic enzymes solution has been added, is shaked at 70 rpm for a time ranging from 3 to 4 hours at a temperature of 30°C; the digestion time is quite variable: it is therefore necessary to supervise the progress of the wall digestion state at

predetermined intervals by taking small aliquots of the solution and look at them with the aid of a microscope.

When the fungal wall appears to be fully digested and the supernatant is composed almost exclusively by protoplasts, digestion can be considered as finished. The solution (still in falcon) is centrifuged at 2000 rpm for 1': from now, onwards the protoplasts have to always be kept at 4°C and it's necessary to work on ice.

After this minute of spinning, all wall components that have been digested and the enzymes themselves are settled as pellets while in the supernatant, which is recovered in new falcons and top off to 50 mL with STC Buffer, are suspended protoplasts.

After a new centrifugation at 3000 rpm for 5 minutes, pour off supernatant and re-suspend in 50mL of STC Buffer (at the end of the protocol). Centrifuge again at same conditions and repeat this step one more time if necessary. After these cleaning steps, supernatant is discarded leaving just a little STC Buffer in the bottom of each tube.

At this point, the protoplasts are isolated and have to be counted by using a hemocytometer or a Thoma counting chamber. If necessary, dilutions have to be made in order to obtain a working concentration of $1x10^7$/mL.

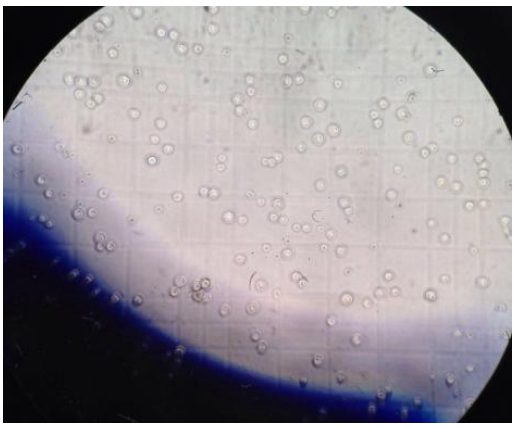


Fig. 17 - AFC-1 protoplasts

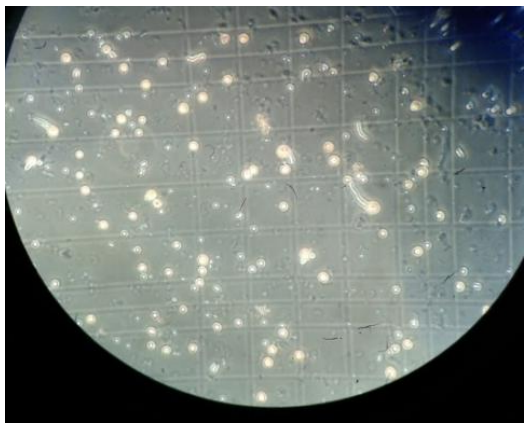


Fig. 18 - Wt protoplasts

After the correct concentration has been reached, protoplasts are incubated with 1-10 µg of DNA of interest due to proceed with the transformation phase. 100 µl of protoplasts are added to 1,5mL tubes for each DNA to be transformed: one tube for the knock-out construct, one for auxotrophic marker and one with no DNA (positive control). Gently pipet and incubate on ice for 20 minutes.

In the following tables, it's explained the different strategies

DNA quantities:

fusion PCR	Linear form	5 µg
Knock-out box	plasmid	10 µg
argD	Linear form	5 µg
Overexpression construct	plasmid	5 µg

The different strategies of transformation can be divided in:

- AFC-1 Knockout with *argD* gene for Zn_2Cys_6
- Knockout mutant (A1 - derived from AFC-1) and Zn_2Cys_6 overexpression construct
- Wt and pyrithiamine antibiotic resistance

In order with an appropriated transformation charges, we used the DNA in linear form altought it's can be possible transform protoplasts using plasmid vectors but with minor efficacy.

After incubation of protoplasts with DNA, 1 mL of PEG 50% is added and left 20 minutes at room temperature.

Finally, 100 µl of each solution containing protoplasts and DNA are plated on various media, as explain above, and incubate the plates at 37 °C for 4-6 days.

Wt and pyrithiamine

Culture media

		CDA		CDA+Pyrithiamine 0.1
Protoplasts only		Positive control		Negative control
Protoplasts + Fusion PCR		Negative control		Selection medium

AFC 1 and *argD* gene (Zn_2Cys_6 Knock out)

Culture media

		MLS		MLS+U		PDA+U
Protoplasts only		Negative control		Negative control		Positive control
Protoplasts+ *argD*		Negative control		Selection medium		Positive control
Protoplasts+ Zn_2Cys_6 KO construct		Negative control		Selection medium		Positive control

MLS is an essential culture medium based on Czapek broth and containing ammonium sulphate 0.4 M that behaves as a ionic and osmotic stabilizing agent: during digestion enzymes eliminate wall components sensitizing fungal protoplasts to the high osmolality of the surrounding environment; the presence of this additive keeps protoplasts alive (Pfeifer & Khachatouriaus, 1987).

From this experiment, 3 putative mutants were isolated: from now on they will be A1, A2 and A3 (Results: Fig.41).

Knockout mutant (A1 - derived from AFC-1) and Zn_2Cys_6 overexpression construct

Culture media

		MLS		MLS+U		PDA+U
Protoplasts only		Negative control		Negative control		Positive control
Protoplasts+ Overexpression construct		Negative control		Selection medium		Positive control

Enzyme Solution (30mL to be divided into 2)

25.5 mL 1.2MNaCl (7g in 100mL diH2O)

3mL 0.2M NaPO4 pH 5.8

0.14g lysing enzyme (4°C) Sigma #L-14122

400µL b-glucorinidase (4°C) Sigma #G-0876

0.08g BSA (4°C dry) Sigma #A2153-100G

1g Vinoflow

50% PEG (100mL)	STC Buffer (500mL)
PEG (MW 4,000) 50g	Sorbitol 109.32g
TrisCl 1M (pH7.5) 1mL	TrisCl 1M (pH7.5) 5mL
$CaCl_2$ 1M 1mL	$CaCl_2$ 1M 5mL
Filter sterilize	Autoclave and store at 4°C

12. MOLECULAR CHARACTERIZATION OF KNOCK-OUT MUTANTS

12.1 Pyrithiamine presence in Wt strain

To verify the integration of Fusion PCR, containing the pyrithiamine resistance, in the hypothetical mutants, grown on selective media with the antibiotic presence, we tested the fungi with PCR targeted to amplify the gene resistance (Results: Fig. 24).

Primers used:

534 Fw	gcgatgcagagagaagaagc	595 bp
Prt I Rev	ccgtatagatcagcggcac	

PCR Conditions:

10X PCR Buffer (Biotaq Bioline)	2.5 µl
$MgCl_2$ (50 mM)	1 µL
dNTP Mix (2,5 mM each)	1 µL
Control PCR Primers (10 µM)	1 µL each
Control DNA Template	1 µl
Taq Polymerase (Biotaq Bioline)	1,25 U
Water	to volume
V_f	25 µL

PCR settings:

Step	Time	Temperature	Cycles
Initial denaturation	4’	96°C	1X
Denaturation	15”	96°C	25X
Annealing	15”	58°C	
Extension	30"	72°C	
Final extension	3’	72°C	1X
Hold		4°C	

See fig. 25 for results

This second PCR was made to verify the correct integration of pyrithiamine instead of $Zn_2Cys_{6.}$

Primers used:

P7	GATATGGTGTGCTCCGAGGA	3011 bp
Prt I Rev	CCGTATAGATCAGCGGCAC	

PCR Conditions:

10X PCR Buffer (Biotaq Bioline)	2.5 µl
$MgCl_2$ (50 mM)	1 µL
dNTP Mix (2,5 mM each)	1 µL
Control PCR Primers (10 µM)	0,5 µL each
Control DNA Template	1 µl
Taq Polymerase (Biotaq Bioline)	1,25 U
Water	to volume
V_f	25 µL

PCR settings:

Step	Time	Temperature	Cycles
Initial denaturation	4’	96°C	1X
Denaturation	15”	96°C	26X
Annealing	15”	58°C	
Extension	3'	72°C	
Final extension	3’	72°C	1X
Hold		4°C	

12.2 Knock-out mutants for Zn_2Cys_6 in AFC-1

12.2.1 PCR

Once singularized (on selection plates MLS+U) their DNA was extracted with the aim to test it with a PCR for the amplification of various regions of the construct of interest, confirming (or not) the correct transformation and recombination event (Results: Fig. 42).

Primers used:

3U4	TTAATTAATTGATCAGGCAAGGTCCTCG
AFZn_P6	TTGATCAGGCAAGGTCCTCG
AFZn_P10	CCATTCGTCCACCATTGCAT
465 FW	CACCCGGATATCCTGACCTC

PCR Conditions:

10X PCR Buffer (Biotaq Bioline)	5 µl
$MgCl_2$ (50 mM)	2 µL
dNTP Mix (2,5 mM each)	1 µL
Control PCR Primers (10 µM)	1 µL each
Control DNA Template	100 ng
Taq Polymerase (Biotaq Bioline)	1,25 U
Water	to volume
V_f	50 µL

PCR Settings:

Step	Time	Temperature	Cycles
Initial denaturation	4’	96°C	1X
Denaturation	30”	96°C	35X
Annealing	15”	59°C	
Extension	2'30"	72°C	
Final extension	10’	72°C	1X
Hold		4°C	

12.2.2 Southern Blotting

Total genomic DNA, extracted according to the protocols described above, was digested at 37°C using the *Eco*RI restriction enzyme (MBI Fermentas) (cleavage site: 5 '- GAATTC - 3') for different times depending on fungal strain (5h for WT, 3h for AFC-1, 3.5h for A1, 5.5h for A2, 3,5h for B, 2h for Arg1, 2h for Arg2).

The choice of the restriction enzyme to be used for genomic DNA digestion falls on proteins, which does not cut within genes of interest; restriction with *Eco*RI can also provide information about the number of copies or alleles of Zn_2Cys_6.

Conditions for the Enzymatic Digestion	
*Eco*RI buffer10X	20 µL
*Eco*RI 10U/µL	3 µL
DNA	5-10 µg
H_2O sterile	up to 200 µL

The digested DNA is subjected to horizontal migration on gel agarose (0,7% agarose in TAE) in an electric field for at least 3 hours at 70V (Results: Fig. 44).

For the labeling of the probe (Zn_2Cys_6) labeled with Digoxigenin (a steroid hapten, ROCHE) which is used for hybridization, DNA extracted from *A. flavus* 3357 wild type strain was amplified in a thermocycler Eppendorf Mastercycler by a simple PCR (Results: Fig. 43).

Primers used for the obtainment of the probe (543 bp):

AF_534 FW	GCGATGCAGAGAGAAGAAGC
AF_534 REV	AGTATACGCCGAGTCAAGGG

PCR Conditions:

10X PCR Buffer (Biotaq Bioline)	5 µl
$MgCl_2$ (50 mM)	2 µL
dNTP* Mix (10 mM each)	1 µL
Control PCR Primers (10 µM)	1 µL each
Control DNA Template	10 ng
Taq Polymerase (Biotaq Bioline)	1,25 U
Water	to volume
V_f	50 µL

PCR settings:

Step	Time	Temperature	Cycles
Initial denaturation	4’	96°C	1X
Denaturation	30”	96°C	26X
Annealing	30”	55°C	
Extension	1"	72°C	
Final extension	5’	72°C	1X
Hold		4°C	

After Electrophoresis of digested DNA, the gel undergoes a series of washes in denaturing solutions (to produce single-stranded molecules ready for hybridization with probe).

After Denaturation phase, agarose gel is ready for an over-night Blotting where DNA is transferred (blotted) from the gel to a nitrocellulose membrane.

Once DNA is transferred, this is fixed on the membrane through a UV-crosslinking.

After the crosslinking, membrane is rinsed quickly in water and then drips.

Next step involves an initial phase of Pre-Hybridization of the membrane carried out in an hybridization oven rotating at 50°C for 2 hours: the membrane is immersed in 20 mL of pre-hybridization solution (DIG Easy Hyb, Roche).

The following step, Hybridization, takes place overnight at 49°C in pre-hybridization solution to which 5-20 ng/µl of probe (marked with Digoxigenin), previously boiled for 10 minutes and cooled on ice for 5 minutes, is added.

It is very important to calculate an appropriate hybridization temperature (T_{hyb}) as this depends on the percentage of G + C residues in probe sequence, on the melting temperature (T_m) and on the length of the probe, according to the following formulas:

$$T_m = 49.82 + 0.41\ (\%\ G + C) - 600/l$$

$$T_{hyb} = T_m - (20° \text{ to } 25°C)$$

The membrane is then washed with two passages in Wash buffer at different SSC concentrations (sodium chloride and sodium citrate): 2X and 0.1X for 10' and 20' respectively (each washing is repeated twice).

The following step consists of other two washings with a Blocking solution: during the second wash the antibody anti-Digoxigenin, conjugated with Alkaline Phosphatase [1: 10000 of anti-dig (Roche) in Blocking solution], is added to the Blocking reagent: the substrate of the antibody is the steroid hapten Digoxigenin and it has the role to reveal the sites where the probe hybridizes.

The membrane is then washed twice with a Washing Detection Buffer; each washing lasts 30 minutes. At the end, a solution containing fluorescent antibody CDP-star (Roche) is added: CDP-star binds to the anti-Digoxigenin and, together with the Alkaline Phosphatase, this complex became luminescent.

The last step concerns the detection of the chemo-luminescence: we used the ChemiDoc™ MP System (Biorad) which is a tool that allows, at predetermined intervals decided by the operator, to detect the signal coming from the membrane (Results: Fig 45).

Solutions for Southern Blotting:

Denaturation solution 0.5N NaOH 1.5M NaCl Autoclave	Neutralization solution 0.5M Tris-HCl pH 7.5 3M NaCl Autoclave
20X SSC Buffer 3M NaCl 300mM Sodium Citrate pH 7 Autoclave	10X SSC Buffer 1,5M NaCl 150 mM Sodium Citrate pH 7 Autoclave
2X Wash solution 2X SSC 0.1% SDS	0.5X Wash solution 0.5X SSC 0.1% SDS
Maleic Acid buffer 100 mM Maleic Acid 150 mM NaCl pH 7,5 Autoclave at 112°C for 40’	Detection buffer 100 mM Tris-HCl 100 mM NaCl pH 9.5 Autoclave
Blocking solution 10X 10% Blocking reagent in Maleic Acid buffer	Blocking solution 1X 1% Blocking reagent in Maleic Acid buffer

12.3 Over-expression -mutants for Zn_2Cys_6 in Knock-out mutant A1

A dozen mutants, which were selected through fluorescence emission (Results: Fig. 40), are tested for the presence of the gene of interest by using a simple PCR program (primers ZnFW1Hind/ ZnR2Kpn) (Results: Fig. 38).

PCR Conditions:

10X PCR Buffer (Biotaq Bioline)	5 µL
$MgCl_2$ (50 mM)	2 µL
dNTP Mix (2,5 mM each)	1 µL
Control PCR Primers (10 µM)	1 µL each
DNA	100 ng
Taq Polymerase (Biotaq Bioline)	1,25 U
Water	to volume
V_f	50 µL

PCR Settings:

Step	Time	Temperature	Cycles
Initial denaturation	4’	96°C	1X
Denaturation	30”	96°C	30X
Annealing	15”	61°C	
Extension	3’	72°C	
Final extension	10’	72°C	1X
Hold		4°C	

12.4. Morpho-physiological parameters of putative AFLA-096370 mutants (Results: Tab. 4)

12.4.1 Morphologicalgrowth

WT, AFC-1, Knock-out, OE and Arg mutants are grown in liquid medium for 10 days in order to acquire information from their morphological behavior (compared to WT and to AFC-1 whose growth is documented).

12.4.2 Conidial count

By an optical microscope and with the aim of characterizing mutants not only from a molecular point of view, conidial counting has been accomplished using the Thoma counting chamber.

Each sample's spores was diluted 1: 200 in sterile distilled water and triton X-100 0.01%.

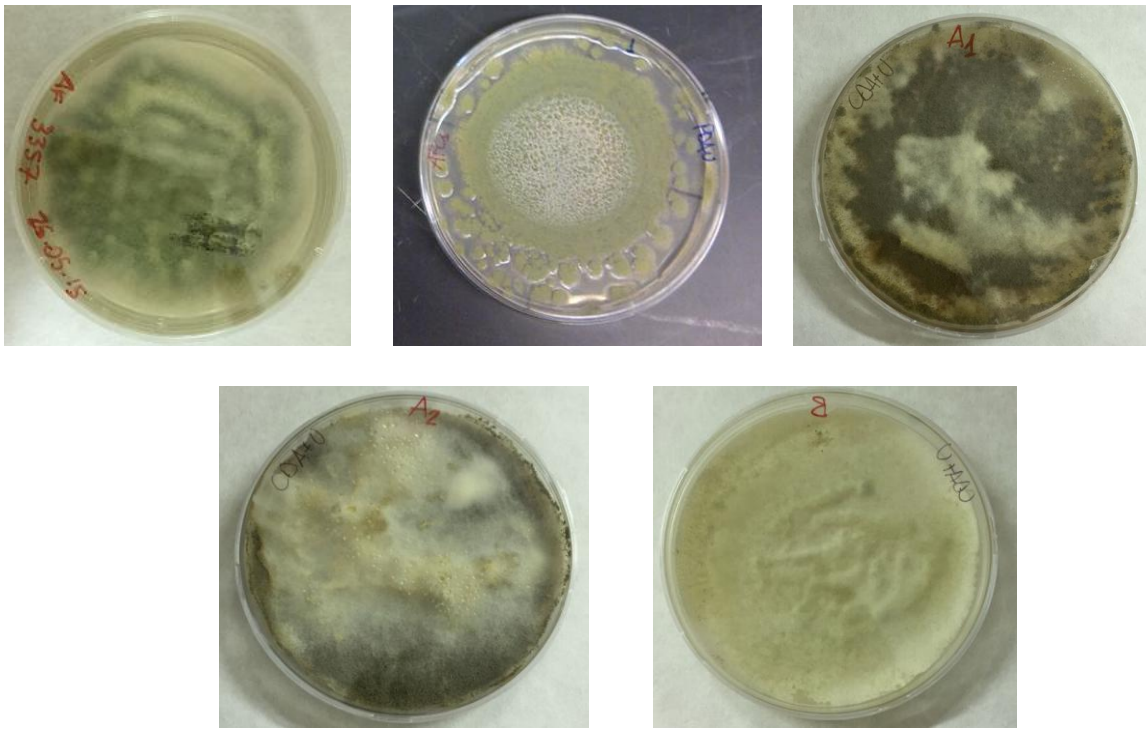


Fig. 19 - WT, AFC-1, A1, A2, B (C32 OE) mutant

12.4.3 Aflatoxin production

After preparing a solution containing chloroform and methanol (2: 1 v/v), 1mL of this is added to 1 mL of culture filtrate (all this takes place in glass conical).

Vortex well and with the help of a Pasteur pipette the lower phase is collected taking care not to bring aliquots of the upper phase. Transfer the mixture to a new conical glass.

Repeat the washing with 1 mL of chloroform: methanol solution and vortex well. Collect only lower phase.

After retrieving all the phase, the samples are evaporated to dryness and then re-suspended in 200 µL of methanol. Aflatoxins suspended in methanol can be evaluated (at first sight) irradiating the samples with a UV lamp. In order to obtain a more precise quantification, samples are transferred into glass vials and subjected to HPLC analysis. Five milliliters of a solution of chloroform:methanol 2:1 v/v, were added to 5 mL of fungal culture filtrates after 15 days of incubation . The solution was spinned at high speed for 1 min and 10mL of the extract were diluted with 40mL of water. Ten milliliters of the diluted solution were filtered through 0.45µm filter and purified by immunoaffinity column (Aflatest, Vicam). Aflatoxin B1 was eluted with 1mL of methanol (Sigma, Milan, Italy), dried and the residue was dissolved with 200µL of methanol. Aflatoxin B1 content was determined by HPLC-DAD equipped with a reversed phase Zorbax-Aq C18 column (150×2.0 mm I.D, 3.5µm) thermostated at 30°. The separations were performed by gradient elution of increasing concentration of acetonitrile (Romil, Cambridge, UK) in water (Romil, Cambridge, UK), both acidified with 1% v/v of formic acid, at a flow rate of 0.2 mL/min. Detection was performed at 363nm. For the quantification of aflatoxin B1 in different matrices, a calibration curve was constructed using standard aflatoxin B1 at different concentrations. The identification and quantification of aflatoxin B1 were performed respectively based on its retention times and spectroscopic spectrum and by the external standard method using a six point regression graph of the UV-visible absorption data collected at 363nm.

12.5 Real-Time qRT-PCR

Starting from 20 mg of lyophilized and grinded (using liquid nitrogen) mycelium (RNA is extracted (Plant/Fungi RNA Purification kit, Norgenbiotek). After quantification (Qubit RNA BR Assay Kit, Life Technologies), complementary DNA (cDNA) was obtained by Retro Transcription.

Retro Transcription Conditions:

Buffer 5X Trans Amp SensiFast (Bioline)	4 µl
RT SensiFast (Bioline)	1 µl
RNA	1 µg
H_2O DEPC	to volume
V_f	20 µL

Retro Transcription Program:

Step	Time	Temp
Pre-Annealing	10’	25°C
Reverse Transcription	15’	42°C
Inactivation	5’	85°C
Hold	10’	4°C

To compare each strain and the respective activities of the genes of interest (Zn_2Cys_6, Sal-OH, NepA, C6 and C_2H_2 TF, *arg*D), we need the qRT-PCR of *A.flavus* β-tub (AFLA_068620), house-keeping gene.

Primers for β-tub		bp	T ann
Af_βtub_for	gtgaccacctgtctccgttt	211	62°C
Af_βtub_rev	ggaagtcagaagcagccatc		
Zn_2Cys_6 TF		bp	T ann
M41TF_FW2	tccaaggaaaattgcctggc	121	65°C
M41TF_REV2	agcagcaggtcacatatggt		
Salicylate hydroxylase		bp	T ann
Sal-OH_FW2	tcgctcctctcatccatcac	133	66°C
Sal-OH_REV2	cggagcggcaattcacttaa		
NPP1 domain protein		bp	T ann
NPP1_FW2	aagacgaacaacccacaacg	134	64,5°C
NPP1_REV2	ggaagagactcccaggcaat		
C6 and C2H2 transcription factor		bp	T ann
M45TF_FW1	gcagtccacatttcccgatc	165	65°C
M45TF_REV1	ggtagaagtctgtgcgggat		
*arg*D		bp	T ann
Arg probe Fw α	tccacgcctcgaacctttat	160	63°C
Arg 600 Rev	ttacgagcgaacttgatggc		

Tab. 2- Primers for q-RT PCR

qRT-PCR conditions:

Primers	0,4 μl (each)
SYBR 2x	5 μl
cDNA	1 μl
Water	bring to volume

V_f =10 μl

qRT-PCR settings:

Step	Time	Temperature	Cycles
Hold stage	10’	95°C	1X
PCR stage	15”	95°C	60X
	15”	T ann (see table)	
	15”	72°C	
Melting stage	15’’	95°C	1X
	1’	65°C	
	15’	95°C	
Hold		8°C	

The statistical analysis was performed three times with similar results. When necessary, acquired data were compared using Student’s t-Test (n=3), P<0,05 (Results: Fig. 46)

13. KERNELS INOCULATION

The maize used is Pioneer P1543 FAO class 600 from 130 days, gently given by “Agricola 200”.

Fungal strains: WT strain (wild-type) Af3357,
AFC-1 strain,
C32 OE mutant,
NepA OE-GUS B5-12 strain
NepA KO-GUS B9-5 strain.

Each sample of kernels (10 in triple) is prepared after two washes with autoclaved water plus bleach 60% for 20 minutes and then it was washed with autoclaved water. The maize was inoculated with a pin bar wet with fungal spores (1 x 10^6 spores/mL).

After 2, 3 and 4 days (grown at 30°C) the kernels were taken and analysed.

13.1 Molecular characterization of inoculated kernels

13.1.1 RT-qPCR

Total RNA was extracted using Trizol (see Materials and Methods 8) and the complementary DNA (cDNA) was obtained by Retro Transcription.

To compare each strain and the respective activities of the genes of interest (Sal OH-salicylate hydroxylase- and NepA- necrosis and ethylene inducing peptide-), we need the qRT-PCR of *A.flavus* β-tub (AFLA_068620), house-keeping gene.

Primers for β-tub		bp	T ann
Af_βtub_for	gtgaccacctgtctccgttt	211	62°C
Af_βtub_rev	ggaagtcagaagcagccatc		
Salicylate hydroxylase		bp	T ann
Sal-OH_FW2	tcgctcctctcatccatcac	133	66°C
Sal-OH_REV2	cggagcggcaattcacttaa		
NPP1 domain protein		bp	T ann
NPP1_FW2	aagacgaacaacccacaacg	134	64,5°C
NPP1_REV2	ggaagagactcccaggcaat		
Quercetin Deoxygenase		bp	T ann
AF_QD_Fw	catcctccgactatgcgtttac	124	62°C
AF_QD_Rev	ctggaagcgacccttgaaat		

Tab.3 Primers for q-RT PCR

qRT-PCR conditions:

Primers	0,4 μl (each)
SYBR 2x	5 μl
cDNA	1 μl
Water	bring to volume

V_f =10 μl

qRT-PCR settings:

Step	Time	Temperature	Cycles
Hold stage	10’	95°C	1X
PCR stage	15”	95°C	60X
	15”	T ann (see table)	
	15”	72°C	
Melting stage	15’’	95°C	1X
	1’	65°C	
	15’	95°C	
Hold		8°C	

The statistical analysis was performed three times with similar results. When necessary, acquired data were compared using Student’s t-Test (n=3), P<0,05

For every strain, were inoculated six kernels as biological replicates and, then, was made three technical replicates in qRT-PCR (Results: Fig 47).

13.1.2 Salicylic acid and Jasmonic acid extraction

The frozen samples (inoculated kernels) were ground under liquid N_2 with mortar and pestle. An amount of 250 mg of the resulting powder was extracted with 750 µl MeOH–H_2O–HOAc (90:9:1, v/v/v) and centrifuged for 1 min at 10,000 rpm. The supernatant was collected and the extraction was repeated. Pooled supernatants were dried under N_2, suspended in 200 µl of 0.05% HOAc in H_2O–MeCN (85:15, v/v), and finally filtered with a Millex-HV 0.45lm filter from Millipore (Bedford, USA). Alternatively, frozen samples were lyophilized and ground with agate mortar and pestle; in this case only 45 mg of the resulting powder was used for the extraction. Quantitation was done by the standard addition method by spiking control plant samples with SA and JA solutions (ranging from 50 to 1000 ng ml^{-1} and from 1 to 20 ng ml^{-1}, respectively). The internal reference standard added for the quantitative analysis was α- naphthalene-acetic-acid (NAA, $C_{12}H_{10}O_2$) from SIGMA, at a final concentration of 1 µM.

13.1.3 HPLC-MS/MS analysis

An amount of Standards of salicylic acid >99% (Fluka, Buchs, Switzer- land) and (±)-jasmonic acid >97% (Sigma–Aldrich, Stein- heim, Germany) were prepared at a

concentration of 500 mg l^{-1} in MeOH. The working SA and JA solutions of 1000 and 20 µg l^{-1}, respectively, were made by diluting the standard solutions with the initial LC mobile phase (0.05% HOAc in H_2O–MeCN, 85:15, v/v).

The equipment, chromatographic column and analysis software were all from Agilent Technologies (Santa Clara, CA, USA). Samples were analysed by LC (HPLC 1200 series rapid resolution) coupled to a triple quadrupole MS (G6420 series triple quadrupole, QqQ; Agilent Technologies) equipped with an electrospray ionization source (ESI). The acquisition was in positive ion mode. Chromatographic separation was performed trough a Zorbax SB-C8 rapid resolution HT 2.1 x 50 mm 1.8 µm 600 Bar column (Agilent Technologies). The elution gradient was carried out with binary solvent system consisting of 0.05% HOAc in H_2O (solvent A) and MeCN (solvent B) at a constant flow-rate of 600 µl/min and a split 1/3. A linear gradient profile with the following proportions (v/v) of solvent B was applied (t (min), %B): (0, 15), (3, 15), (5, 100), (6, 100), (7, 15), (8, 15) with 5 min for re-equilibration.

The MRM analysis was performed as below:

Hormones	Precursor ion	Fragmentor	CE (V)	Product ion	Ritention time
NAA	245	100	16	180,8	6,469
Jasmonic acid	209,2	135	28	59,1	5,682
Salicylic acid	137,2	135	20	92,9	5,284

13.2 Histological Protocol

The samples was covered with the ethanol 70% for two hours and then replace with ethanol 90% for two hours and next ethanol 100% overnight. Two hours in resin (Technovit® 7100-Haraeus-Kulzer Germany)/absolute ethanol (1/3); two hours in resin/ absolute ethanol (1/1); two hours in resin/ absolute ethanol (3/1). In overnight, at 4 °C, resin plus catalyst 1 and then, for several hours, replace with resin plus catalyst 2 (20:1) at room temperature. Now, the included section can be cut with Microtom HM 350 with 8 nm as thickness, colorated with toluidine blue and visualized.

13.3 GUS Histochemical Assay Protocol

Transfer samples in acetone 80% (dilution with distilled water) at temperature of -20 °C and punt in -20 °C fridge for 20 minutes. Then remove acetone and wash three times with distilled water and add GUS buffer until the samples are covered. Infiltration for 15 minutes in the machine vacuum and incubation at 37 °C, in darkness for several times (2h, 4h, overnight or more if necessary). Finally, remove GUS buffer, replace with ethanol 70% and preserve at 4 °C in ethanol 70%.

GUS buffer:

- 10 mg X-GlcA (sodium trihydrate; Duchefa-Biochemie)
- 100 µl triton X100 10%
- 200 µl EDTA 0.5 M pH 8.0
- 125 µl potassium ferrocyanide 40 mM (final conc. 0.5 mM)
- 125 µl potassium ferricyanide 40 mM (final conc. 0.5 mM)
- Bring to final volum of 10 mL with Na- phosphate buffer (Na_2HPO_4/NaH_2PO_4) (final conc. 0.1 M) pH 7.0
- Stored at – 20 °C

For 200 mL of Na-phosphate buffer 0.1 M pH 7.0 :

- 39.0 mL NaH_2PO_4 0.2 M
- 61.0 mL Na_2HPO_4 0.2 M
- 100 mL distilled water

14. RUTIN DEGRADATION

A. flavus strain NRRL 3357 was maintained on Czapek Dox Agar (CDA), amended with ZnSO4(5 mg/L) and NaMoO4 (1mg/L) at 30°C. Seven day later the spores were collected putting 5-6 mL of distilled and sterile water and Triton X-100 (0,01%, Sigma) and are inoculated into 100 mL of PDB (1 x 10^5 spores/mL) and grown at 28°C for 7 days in static; the same growth culture of NRRL 3357 was started using 100 mL of PDB containing rutin 0,02 mg/mL (Chitarrini et al. 2014). Mycelia were sampled at 7 different time intervals from 0 up to 168 hours post inoculation (hpi).

14.1 Rutin, Quercetin and Aflatoxin extraction from culture media

2mL of ethyl acetate are added to 2 mL of culture filtrate.

The internal reference standard for the quantitative analysis was margaric acid (17:0, $C_{17}H_{34}O_2$) from SIGMA, at a final concentration of 10 µM for Aflatoxin B1 detection; for Rutin and Quercetin (and their derivative *2*-protocatechuoylphloroglucinol carboxylic acids) the internal reference standard was Chlrogenic Acid ($C_{16}H_{18}O_9$) from SIGMA at a final concentration of 10 µM.

Vortex well, centrifuge for 5 min at 10,000 rpm and with the help of a Pasteur pipette the upper phase is collected taking care not to bring aliquots of the lower phase.

Transfer the mixture to a new conical tube. Repeat the washing with 2 mL of ethyl acetate.

The samples are evaporated to dryness and then re-suspended in 200 µL of methanol, and finally filtered with a Millex-HV 0.45lm filter from Millipore (Bedford, USA).

14.2 HPLC-MS/MS Analysis

The equipment, chromatographic column and analysis software were all from Agilent Technologies (Santa Clara, CA, USA). Samples were analysed by LC (HPLC 1200 series rapid resolution) coupled to a triple quadrupole MS (G6420 series triple quadrupole, QqQ; Agilent Technologies) equipped with an electrospray ionization source (ESI). The acquisition was in positive ion mode. Chromatographic separation was performed trough a Zorbax SB-C8 rapid resolution HT 2.1 x 50 mm 1.8 µm 600 Bar column (Agilent Technologies). The elution gradient was carried out with binary solvent system consisting of 5mM NH4AcO in H_2O (solvent A) and 5mM NH4AcO in MeOH (solvent B) at a constant flow-rate of 600 µl/min and a split 1/3. A gradient profile with the following proportions (v/v) of solvent B was applied (t (min), %B): (0, 0), (14, 100), (18, 100), with 2' for re-equilibration.

The MRM analysis was performed as below:

Compounds	Precursor ion	Product ion	CE (V)	Fragmentor
Rutin	610.9	303.1	48	165
Quercetin	303.1 303.1	228.8 152.9	37	100
2-protocatechuoylphloroglucinol carboxylic acids	291.3 291.3	129.1 112.8	38	90
Aflatoxin B1	313.2	241.1	38	135

RESULTS AND CONCLUSIONS

A. flavus is considered a negative filamentous fungus because of its capacity to produce aflatoxin, with witch contaminate food in pre- and post-harvest, and to cause human diseases. Furthermore, *A.flavus* produce a whole array of secondary metabolites in addition to aflatoxins, and this unknown secondary compounds can be explored for possible and different uses (Cleveland et al., 2009), from pharmaceutical use to turning waste plant polymers into biofuels. Secondary metabolites genes are usually clustered in the genome, each containing enzymatic genes and often transcriptional factors for compounds synthesis (Amaike and Keller, 2011). The SMURF (Secondary Metabolites Unknown Regions Finder) analysis of *A.flavus* genome has predicted 56 clusters (Chang and Ehrlic, 2013). Some clusters are differently regulated in *A.flavus* during its interaction with maize, among which cluster 11, 32, 45, 47, 54 (aflatoxins) and 55 (CPA- cyclopiazonic acid) results strongly up regulated (Reverberi et al., 2013). The regulation of AF biosynthesis is complex and involves several interconnecting networks. AF was one of the first fungal secondary metabolites shown to have all its biosynthetic genes organized within a DNA cluster. These genes, along with the pathway specific regulatory genes *aflR* and *aflS*, reside within a 70kb DNA cluster. These two genes are located divergently adjacent to each other within the AF cluster and are involved in the regulation of AF/ST gene expression, in particular the gene *aflR* encodes a sequence-specific DNA-binding binuclear zinc cluster protein (Zn_2Cys_6) required for transcriptional activation of most, if not all, of the structural genes (Georgianna and Payne, 2009). Beside, most of the gene involved in the biosynthesis of aflatoxin were identified and was obtain evidence for a complex interaction between environmental and nutritional factors and the genetic characteristics of the fungi: an example is represented by zinc. Zinc is essential for aflatoxin biosynthesis and the authors found that the failure of aflatoxin production in Zn-deficient medium was also due to the repression of AflR formation; in fact, Zn serves as a prosthetic group of many enzymes and effects both the primary and the secondary metabolism (Liu and Chu, 1998).

During pathogenic growth there are changes in expression of several genes encoding secondary metabolites. More highly expressed are NepA (Necrosis and Ethylene –inducing Peptide), which belongs to a family of NLP genes; these genes encode effectors required for pathogenicity in some organism, and recent data show that they also may play diverse roles in the pathogen including development (Reverberi et al., 2013).

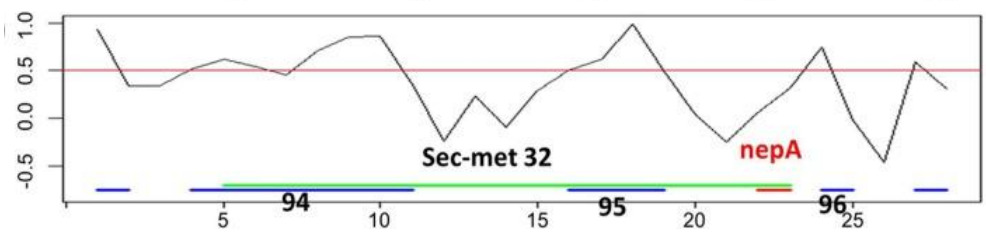


Fig. 20 - Plot of the co-expression of the neighboring genes - Secondary metabolite clusters from Georgianna *et al.* (2010), identified by SMURF algorithm, are denoted by green bars. The *nepA* gene is indicated, by red bar (Reverberi *et al*., 2013

Cluster 32 co-expression with NepA could be signifying that this cluster is activated during the pathogenic process. Also, cluster 32 contains a gene coding for a Salicylate hydroxylase enzyme that degrades salicylic acid. This compound is a plant hormone known to be a critical factor in activation of plant defences in response to pathogen attacks (Ambrose et al., 2015). However, it's still not understood the molecular basis of salicylate hydroxylase-salicylic acid interactions.

1. CHARACTERIZATION OF PYRITHIAMINE MUTANTS FOR Zn_2Cys_6 KNOCK-OUT

The Fusion PCR starts with the creation of all single fragments, 5' UTR region, pyrithiamine gene and 3' UTR region. Each component was amplified (on the left) and purified on column (on the right).

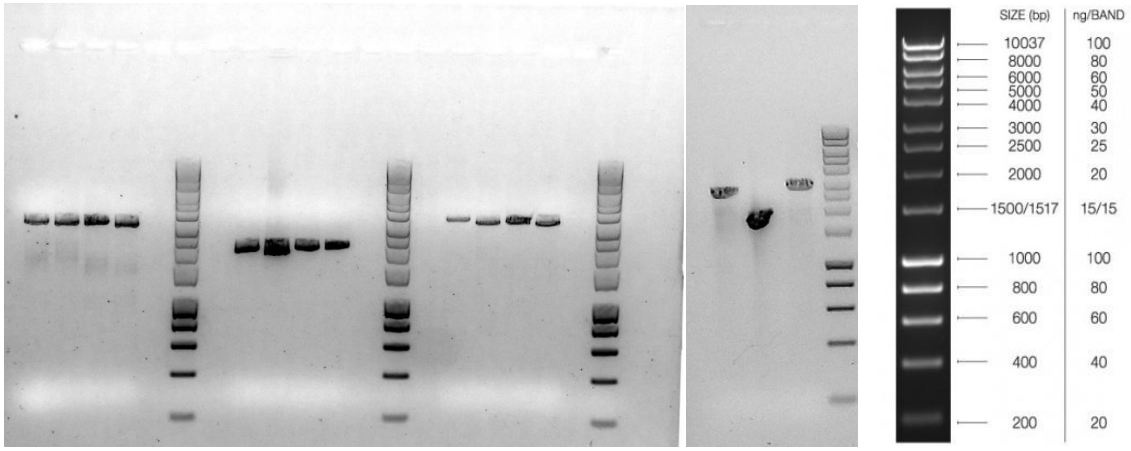


Fig. 21 - Amplification of single fragments: 5'UTR (2872 bp), Pyrithiamine (2000 bp), 3'UTR (3038 bp). DNA Ladder: DNA hyper ladder 1 KB (Bioline).

In the second step the 5'UTR fragment is bound to the pyrithiamine, using primers Zn_Pt_P1/Ptr Rev. The fragments, fused and amplified, are almost 4000 bp long (3970 bp).

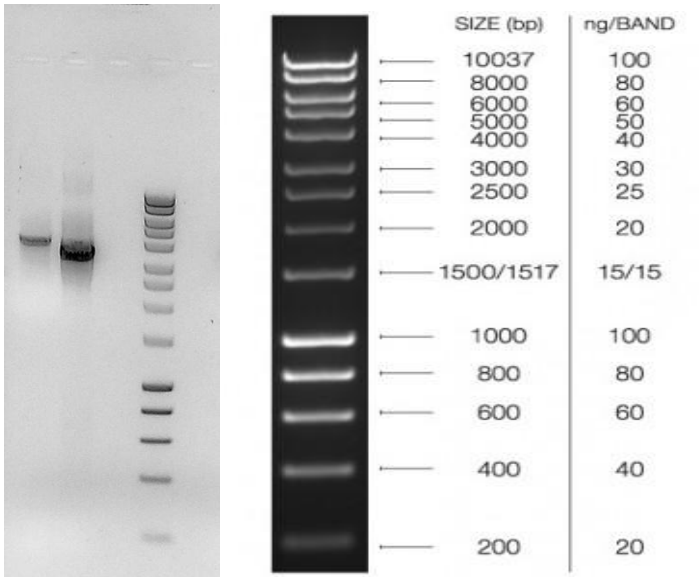


Fig. 22 - Amplification 5'UTR + Pyrithiamine. DNA Ladder: DNA hyper ladder 1 KB (Bioline).

In the last step, 5'UTR + pyrithiamine gene (purified on column) is fused with 3' UTR using the second 'nested' primers (Zn_Pt_P6). The fragments, fused and amplified, are almost 6000 bp long (5784 bp), as the figure 45 shows.

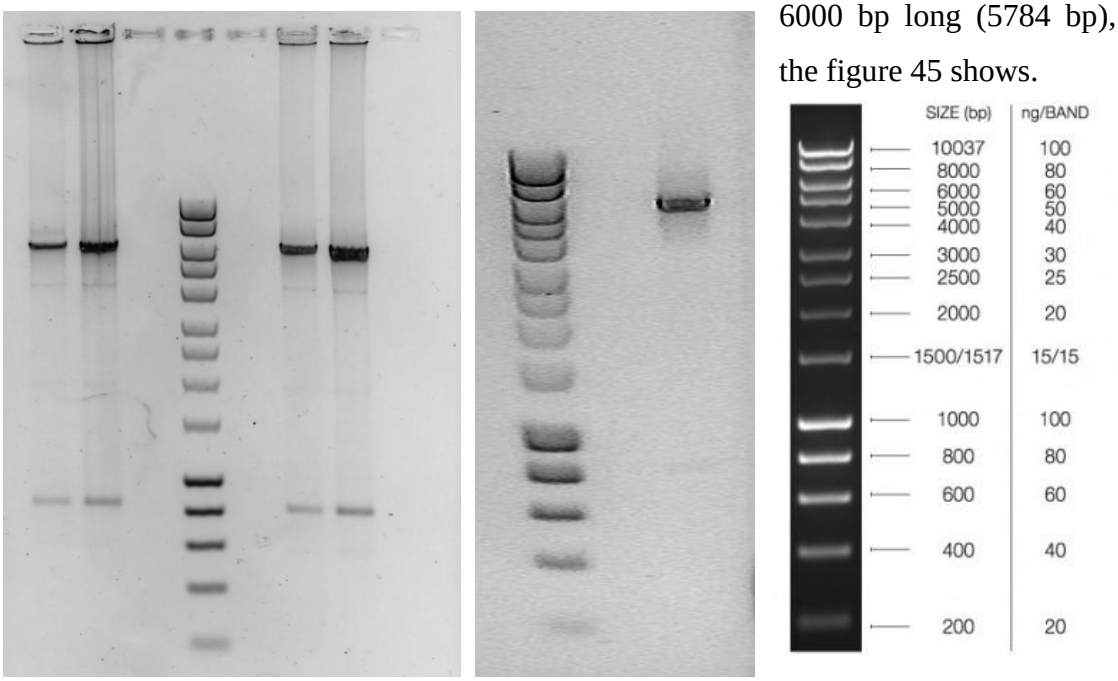


Fig. 23 - Fusion PCR for Zn2Cys6, on the left; after column purification, on the right. DNA Ladder: DNA hyper ladder 1 KB (Bioline).

Now, we obtain our DNA to use to make Zn_2Cys_6 deletion.

After 3-4 days from transformation, hypothetical mutants grown up on selective medium.

Fig. 24 - Hypothetical mutants on CDA+ pyrithiamine

Even they are resistant to pyrythiamine, their growth are different as shown in figure 24.
After the DNA extraction, the hypothetical mutants were tested to the pyrithiamine presence to verify the antibiotic resistance and the integration inside *A.flavus* genome.
In figure 24, it's represented the first screening between the ones that have maintained the resistance and the ones that have lost it during alternated steps on media selective and media not selective.

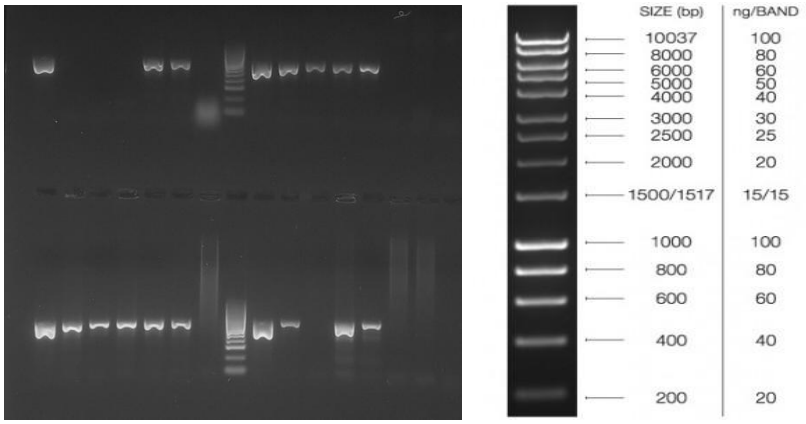


Fig. 25 - PCR for pyrithiamine presence. DNA Ladder: DNA hyper ladder 1 KB (Bioline).

To verify the deletion of Zn_2Cys_6 replaced by pyrithiamine, we made a second PCR using primers P1/Ptr I Rev but the result tell us that the integration is ectopic (Data not shown because of absence of amplification).

2. CREATION OF KNOCK-OUT AND OVEREXPRESSION AFC-1 MUTANT IN AFLA_096370 (Zn_2Cys_6)

AFC1 is an auxotroph for two-principle amino acid: arginine and uracil, so for its growth are needed both in culture media. To choose between the transforming ones and the ones that are not transforming, only colonies that grow up on MLS+U could be considered hypothetical mutants because, as we say before, without both amino acids AFC 1 cannot grow.

First, through TOPO TA cloning approach, two constructs were assembled and later these have been used to transform the AFC-1 strains.

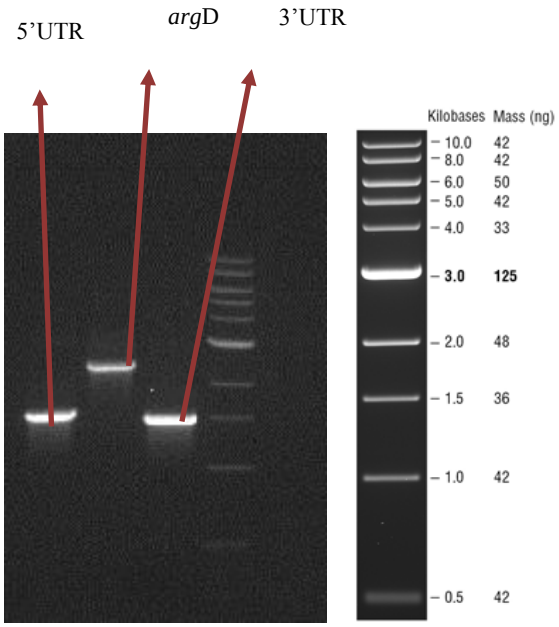


Fig. 26 - Amplification of single fragments using primers 5U1/5U2 for 5'UTR, ARG FW/ARG REV for ArgD and 3U3/3U4 for 3'UTR. DNA Ladder 1KB BIOLABS.

In order to produce a knock-out construct of Zn_2Cys_6 transcription factor (AFLA_096370), 5'UTR and 3' UTR and *argD* (AFLA_135340) gene were amplified starting from DNA of *A. flavus* NRRL 3357 through an extraction protocol from spores.

The following figure refers to the gel photo of PCR control for amplification of single fragments.

In order to exploit events of homologous recombination between Zn_2Cys_6 gene and the knock-out construct, the selectable marker *argD* gene has specifically to be flanked by the 3'UTR and the 5'UTR.

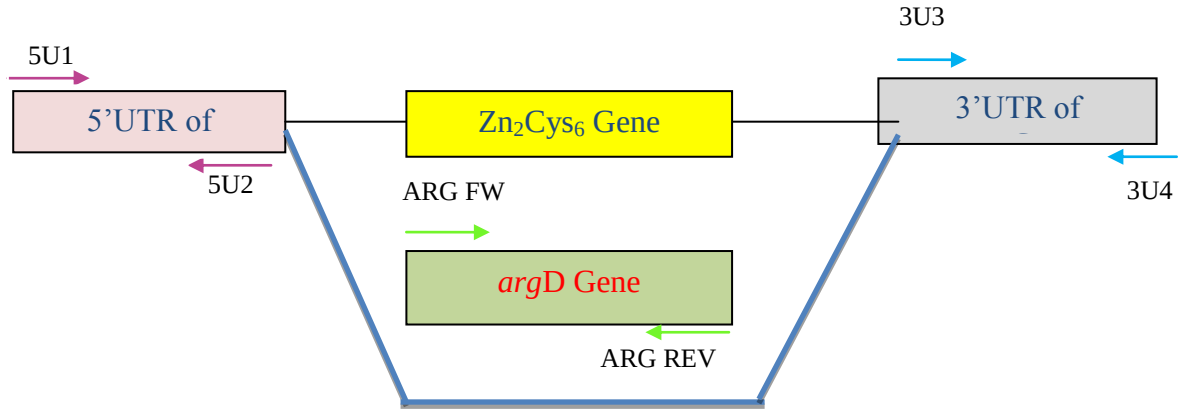


Fig. 27 - Schematic representation of Knock-out construct (Zn_2Cys_6)

The following step consists on cloning each single fragment, amplified from PCR and then purified, in pCR 2.1 vector.

Colonies that have successfully been transformed with plasmids in which each fragment had been cloned, are selected *via* blue/white screening. White colonies are then isolated and plasmids are recovered. In order to control the actual insertion of fragments, we checked using a simple PCR.

Fig. 28 - Screening for colonies *via* blue/white selection

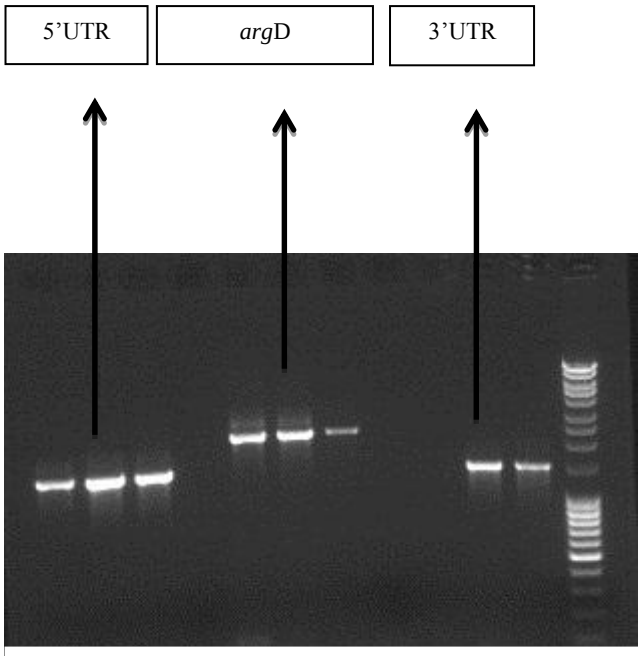


Fig. 29 - Control PCR for the insertion of single fragments into pCR 2.1 vector using primers 5U1/5U2 for 5'UTR, ARG FW/ARG REV for *arg*D and 3U3/3U4 for 3'UTR

Plasmids containing *argD* and the ones containing 5'UTR are digested and separated by gel electrophoresis: bands of interest are then cut and purified.

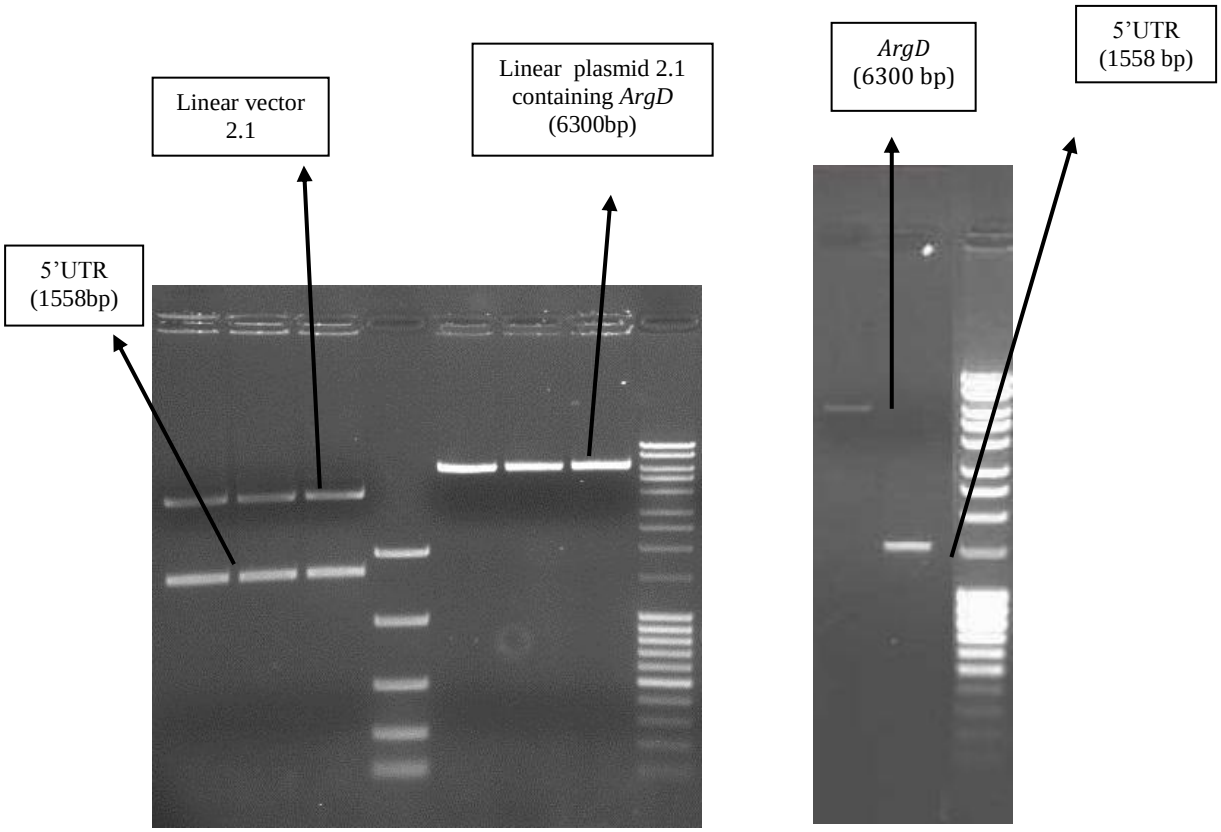


Fig. 30 - Vector 2.1 + 5'UTR and Vector 2.1 + *argD* after double digestion (left) and after purification

DNA so purified is incubated with T4 DNA *Ligase* in a Ligation step with the aim of fusing 5'UTR to vector 2.1+*argD*. A PCR control is necessary to be sure of the insertion event.

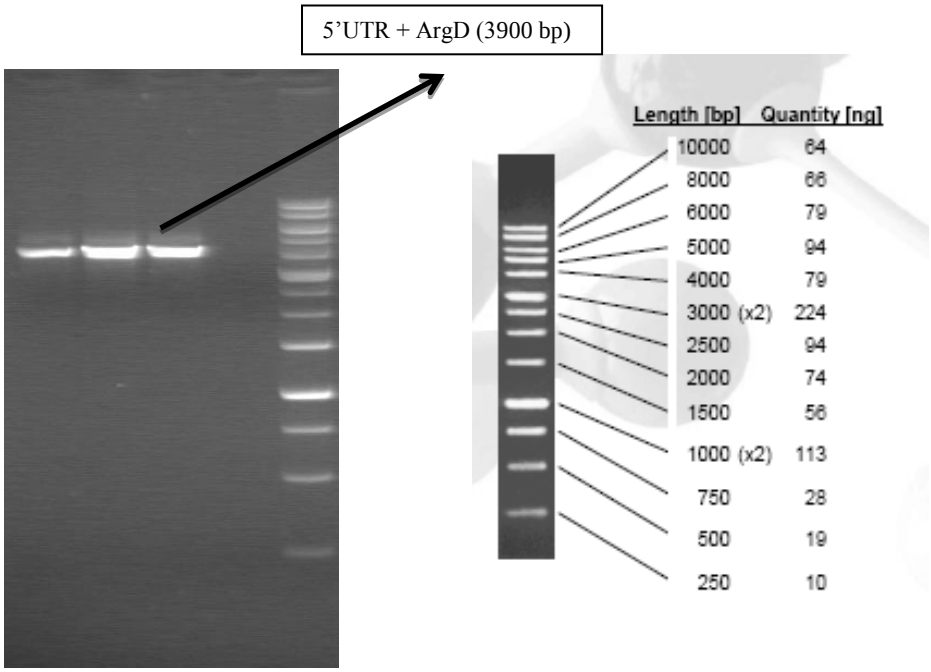


Fig. 31 - Amplification of 5'UTR + *argD* using primers 5U1/ARG REV. DNA Ladder 1KB APPLICHEM

After the 5'UTR insertion into the vector has been verified, we proceeded with a new double digestion using *Sbf*I-HF and *Pac*I enzymes.

At the end of the Digestion phase, DNA is firstly checked by gel electrophoresis and secondly bands of interest are cut and purified.

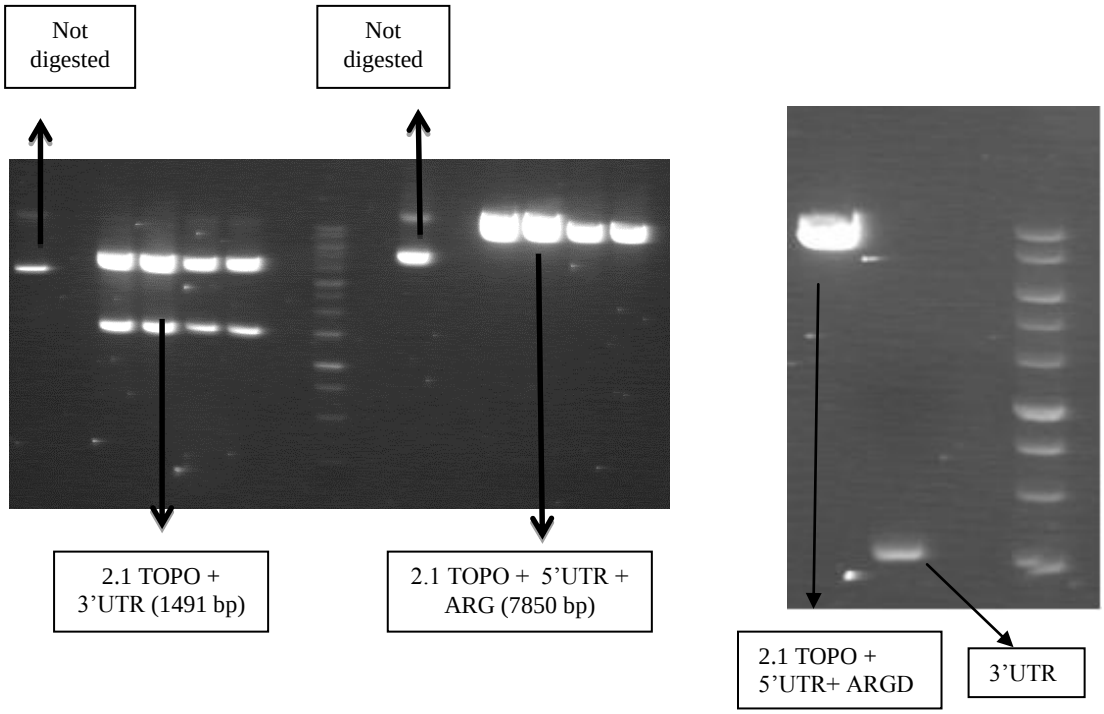


Fig. 32 - Gel electrophoresis of pCR 2.1+ 3'UTR and pCR 2.1+ ArgD + 5'UTR after double digestion (left); and after purification (right)

Once fragments of interest (pCR 2.1 vector + 5'UTR + *argD* and 3'UTR) are obtained, these are incubated with the T4 DNA *Ligase* enzyme to finally get the whole construct.

The digestion of pCR 2.1 vector +5'UTR+*argD*+3'UTR with *Eco*RI and successive PCR control of the colonies selected as positives, confirmed results obtained.

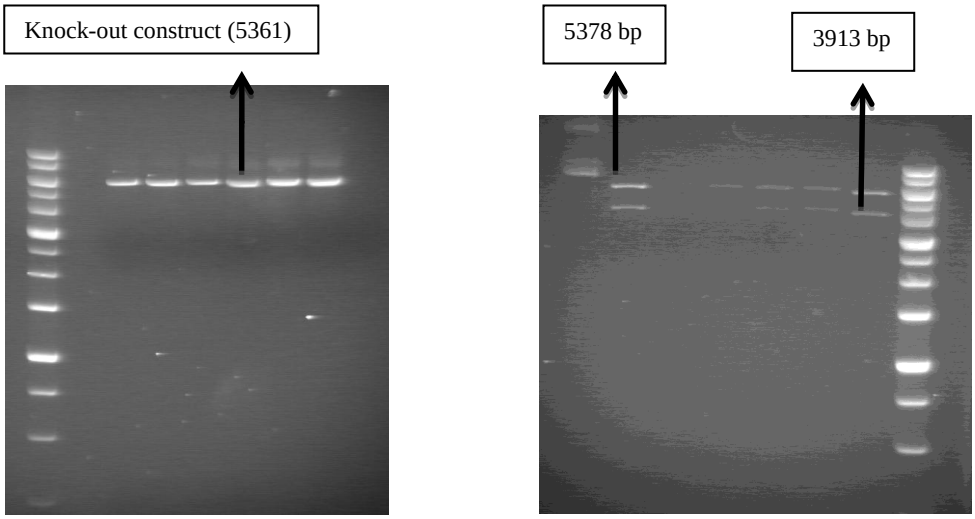


Fig. 33 - Control PCR using primers 5U1/3U4 (left) and digestion with EcoRI (1,5h) (right) – DNA Ladder 1KB APPLICHEM

After attempting the transformation several times, we were able to isolate three mutants hypothetically carrying our knock-out construct (A1, A2 and A3) and two mutants, Arg1 and Arg2, in which the *argD* function had been restored.

The parallel transformation with *argD* has the purpose to demonstrate that the recovery of the production of Acetylornithine Aminotransferase by the mutant is indeed responsible for their survival on a medium devoid of the above aminoacid.

All of these mutants were screened by their ability to grow on a medium in which Arginine has not been provided.

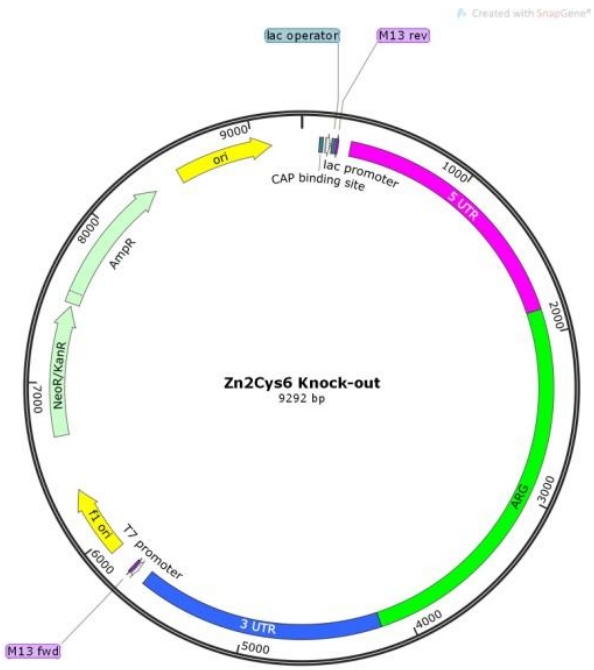


Fig. 34 Schematic representation of pCR 2.1 vector containing 5'UTR+ArgD+3'UTR construct

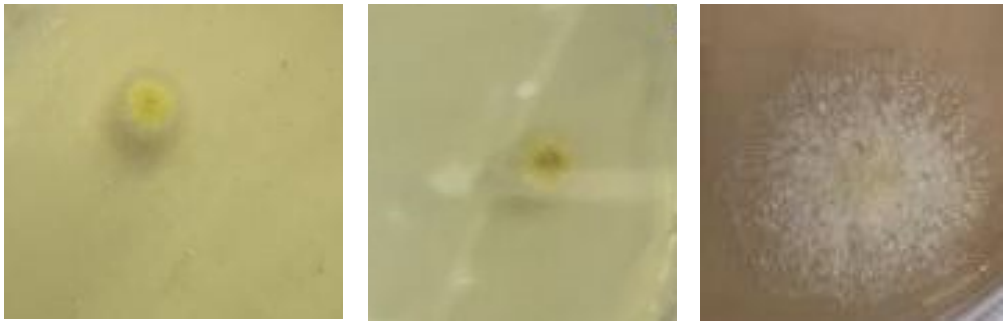


Fig. 41 - Hypothetical knock-out mutant A1, A2 and A3 growing on selection medium MLS+U

Putative knock-out mutants were singularized keeping them on selection medium (CDA+U) and their DNA was extracted in order to be characterized from a molecular point of view. Results obtained from PCR amplification confirmed the insertion of knock-out construct in the genome of A1, A2 and A3.

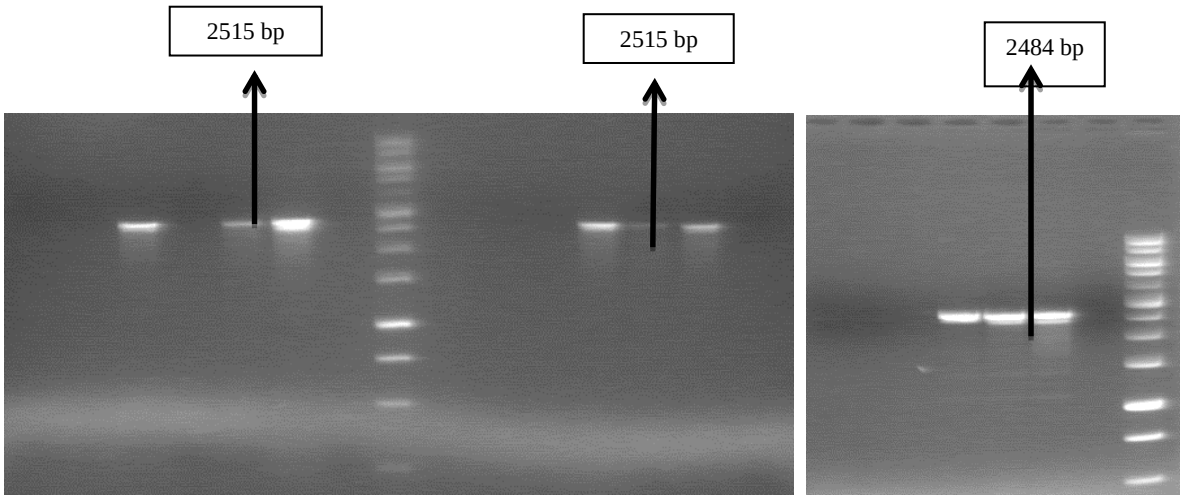


Fig. 42 - PCR control gel photo of amplification of knock out construct fragments.
a) Primers 465 FW/3U4 and 465 FW/Zn_P6 (left) b) Primers 465 FW/Zn_P10 (right)
Loading order: WT, AFC-1, A1, A2, A3, pCR 2.1 vector + 5'UTR+*arg*D+3'UTR. Ladder 1KB Applichem

In order to confirm the positive outcome of the processing and the correct insertion of the construct, we resort to a molecular technique, Southern Blotting, which allows revealing specific DNA sequences in a complex mixture by hybridization of DNA of interest with a labeled probe (Zn_2Cys_6 fragment, 534bp) and screening by chemo-luminescence.

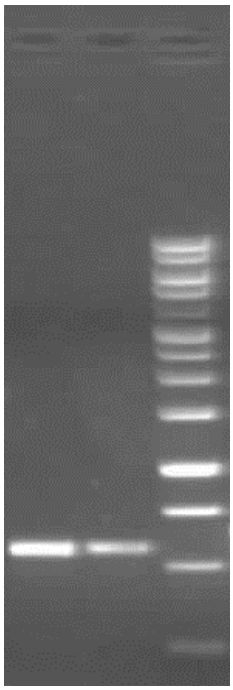


Fig. 43 - Gel electrophoresis f Zn2Cys6 probe for Southern Blotting, after purification (534 bp). DNA Ladder 1KB Applichem

Southern Blotting starts with a genomic digestion (with *Eco*RI): this is a crucial step and it is very important to set appropriately digestion times for each sample.

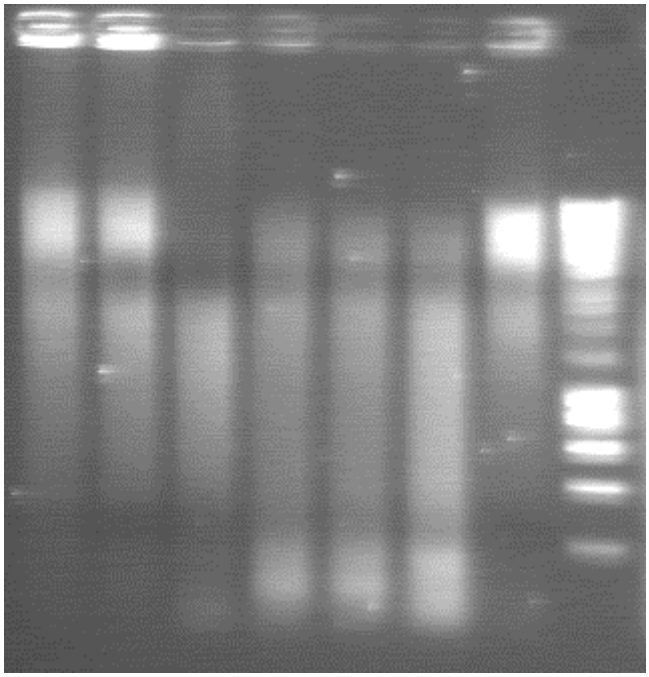


Fig. 44 - Gel electrophoresis of genomic digestion with *Eco*RI.
Loading order: WT, AFC-1, A1, A2, A3, Arg1, Arg2. Ladder 1KB Applichem

Southern Blotting analysis shows a strong signal for Zn_2Cys_6 from A2 mutant whereas A1 and A3 have a weak probe signal.

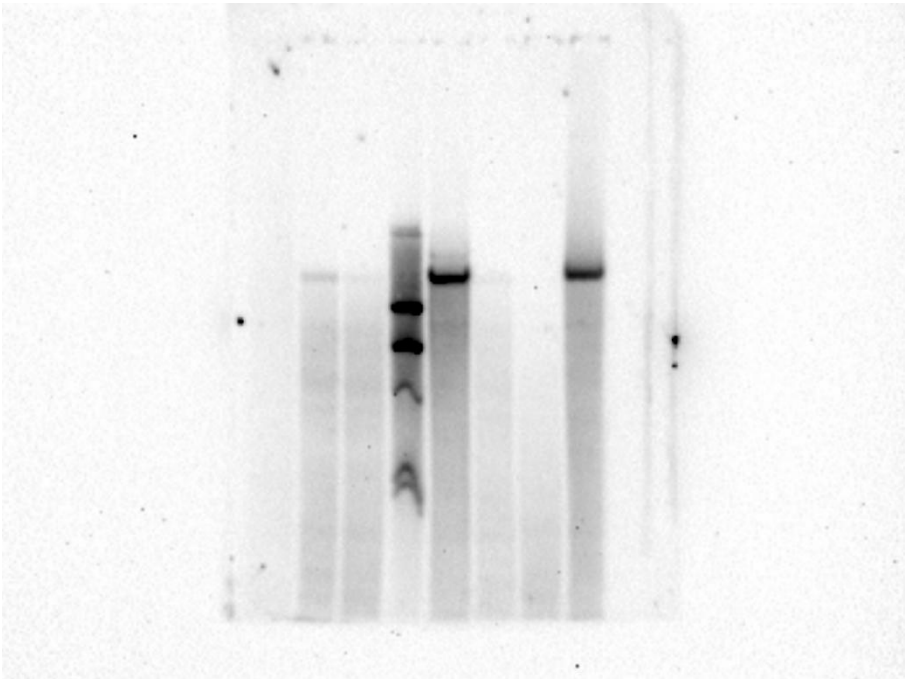


Fig. 45 - Nitrocellulose membrane taken with ChemiDoc™ MP System (Biorad). Loading order: Arg2 - Arg1- B – DNA Molecular Marker II (Roche) – A2 – A1 – AFC-1 – WT. Acquisition after 5 minutes of exposition.

In order to produce an overexpressing mutant AFLA_096370 (Zn_2Cys_6), a region of 2771bp containing the coding sequence of Zn_2Cys_6 (2531 bp) and flanked by 58 bp upstream and 182 bp downstream, has been selected, amplified, and purified on column.

After the Ligation of Zn_2Cys_6 in pCR 2.1. TOPO vector, transformation of competent TOP10 cells and selection *via* blue/white screening, plasmids containing the gene of interest are recovered and checked with a simple PCR.

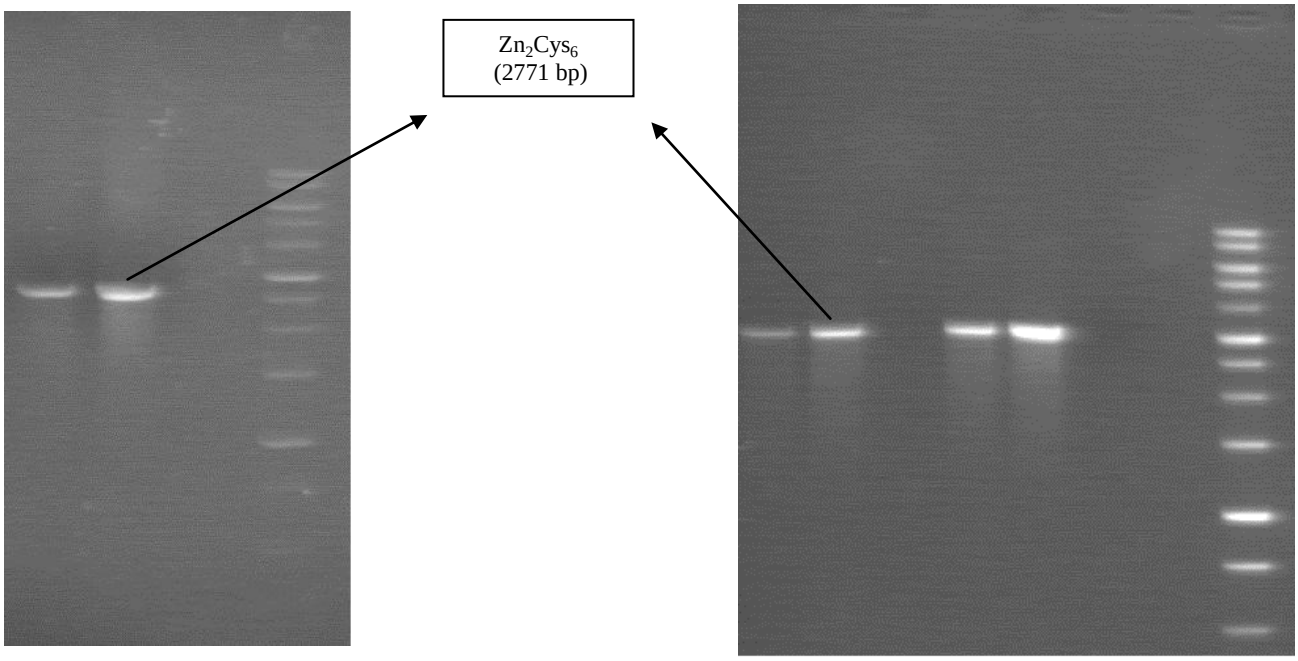


Fig. 35 - Gel electrophoresis of the PCR amplification of Zn_2Cys_6 after purification using GenElute™ PCR Clean-Up, Sigma (on the left) and Zn2Cys (2771 bp) amplification from pCR 2.1 vector (on the right).

With positive results confirmed by PCR, pNuc'Em2 vector and pCR 2.1 vector+Zn_2Cys_6 are double digested with specific enzymes (HindIII and KpnI).

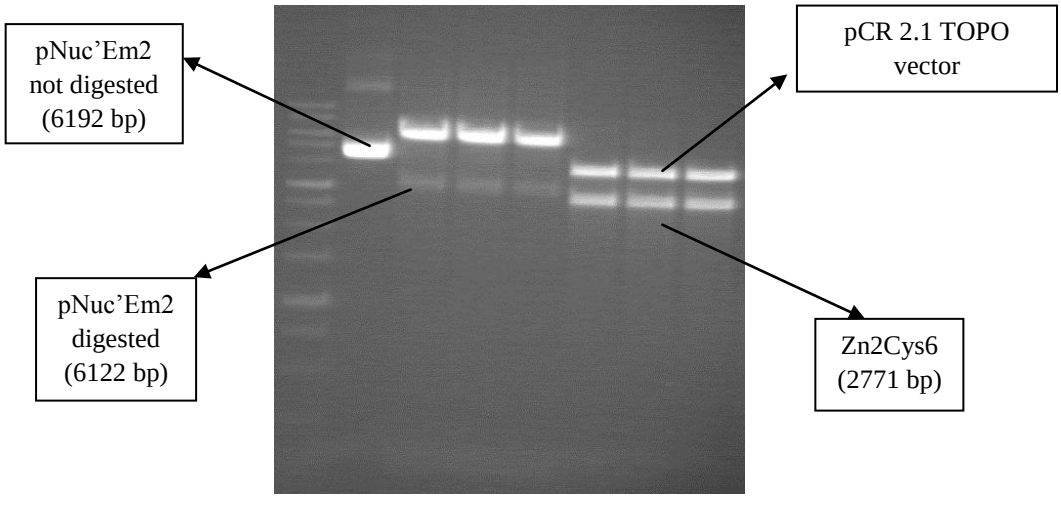


Fig. 36 - *Hind*III and *Kpn*I double digestion gel photo

DNA is purified and Zn_2Cys_6 is cloned into pNuc'EM2 (after Ligation).

Plasmid are then used for for transformation and positive colonies are then tested with a simple PCR.

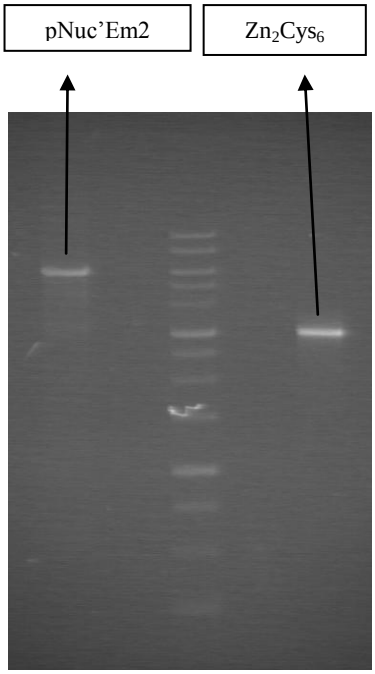


Fig. 37 - Gel of purification: pNuc'Em2 and Zn_2Cys_6

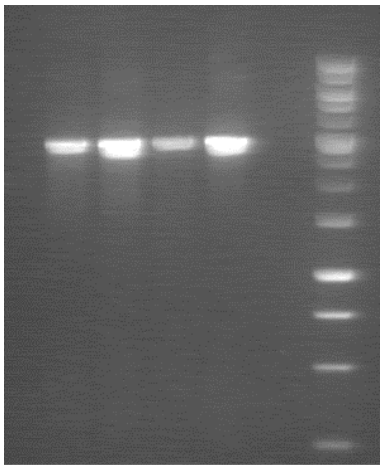


Fig. 38 - Gel photo of PCR amplification of Zn_2Cys_6 (2771bp). Loading order: WT, AFC-1, AFC-1, pNuc'Em2+Zn_2Cys_6, K^-

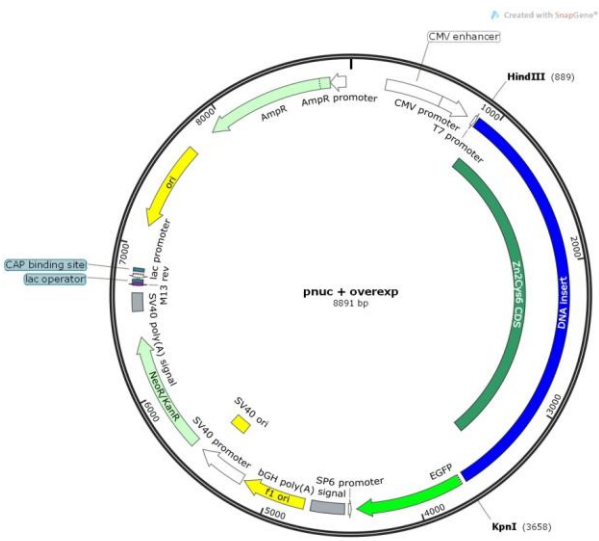


Fig. 39 - pNuc'Em2 + Over-expression construct

Transformation processing of A1 Knock out mutant (form Zn_2Cys_6 deletion) using pNuc'Em2 + Over-expression construct, has led to the isolation of 15 hypothetical over-expression mutants, which were selected through fluorescence emission: the enhanced-GFP has a single absorption peak at 488 nm while the emission of light takes place at 509 nm.

Care must be taken in discriminating between a small fluorescence signal naturally emitted from the fungal walls and a stronger emission that is rather localized predominantly in conidia (Du *et al*., 1999).

Among these overexpression mutants B was selected for for the following physiological and molecular characterizations

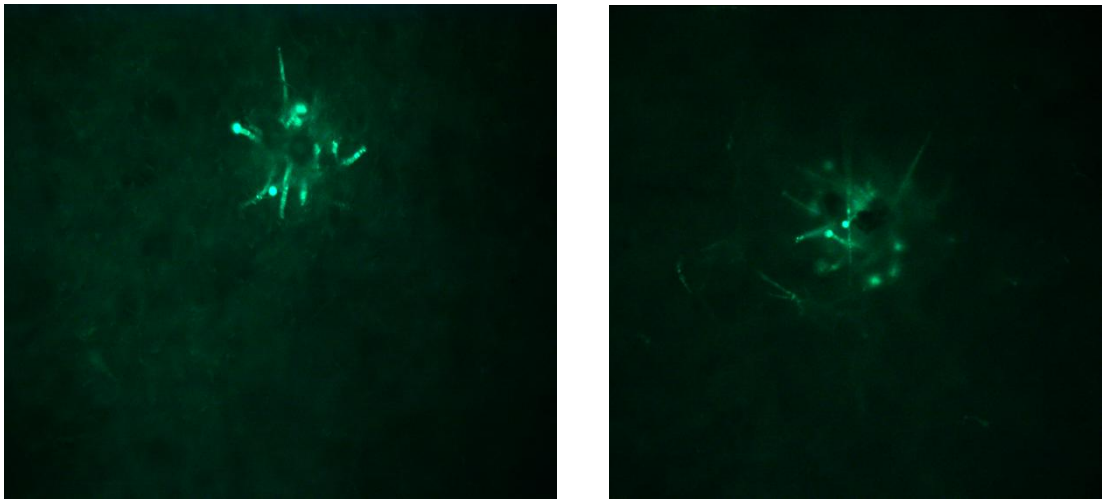


Fig. 40 - Picture of over-expression mutants taken by fluorescence stereomicroscope

3. MORPHOLOGICAL, PHYSIOLOGICAL AND MOLECULAR PARAMETERS OF PUTATIVE AFLA_096370 MUTANTS

3.1 Morphological growth for Zn_2Cys_6 mutants

WT and AFC-1 (in PDB and PDB+A+U) have a similar growth even if WT is faster and produces a huge amount of conidia. A1 shows a similar behavior of growth compared to A2 (on CDB+U): both produce plenty of mycelia and not so many spores. A2, interestingly, present a rust-colored fouling on the walls of the flasks which can be constituted of secondary metabolites specifically produced by this mutant.

B apparently shows a suffering growth but produces lots of conidia: medium looks very turbid.

Arg mutants grow very fast (like WT) and produce plenty of mycelia but not so many conidia. The recovery of Arginine production increases rate of growth.

3.2 CONIDIAL COUNT	(conidia/mL)
WT	$7,5 \times 10^6$
AFC-1	5×10^6
A1	$4,25 \times 10^6$
A2	$3,9 \times 10^6$
B (C 32 OE)	6×10^6

These results show that A1 and A2 have a decreased conidial production while B mutant produces a rather high number of conidia when compared to the two others.

3.3 AFLATOXIN PRODUCTION

Although Zn_2Cys_6 is not directly involved in the biosynthesis of aflatoxins, it is plausible that its deletion might have had a more or less sensitive effect even on this pathway and therefore have altered the entire Secondary Metabolism.

For this reason a quantitative analysis of aflatoxin, produced by the various mutants and released into the culture filtrate, was conducted.

At first detection, by the radiation of aflatoxins suspended in methanol with a UV lamp, it appears that the mutants' filtrate contains a less amount of aflatoxin instead appreciated in WT, in AFC-1 and in Arg mutant filtrate (where the quantities are similar). B mutant, for example, shows a very few accumulation of aflatoxins in his filtrate whereas A2 filtrate seems even poorer.

	mg/mL	Conidia/mL	AFB1 (ppb)
WT	100 %	100%	100 %
AFC-1	90%	67%	90%
A1	60%	60%	20%
A2	55%	50%	10%
B (OE32)	30%	80%	20%

Tab 4 - Morphological growth, Conidial count and Aflatoxin production of putative Zn_2Cys_6 mutants

3.4 q-RT PCR

To clear up these results, we performed cDNA amplification of AFLA_096370 together with a subset of genes, of particular relevance, inside the cluster.

Affy id	Description
2541.m00048	Salicylate hydroxylase, putative
2541.m00045	C_6 and C_2H_2 transcription factor
2541.m00044	C_6 and C_2H_2 transcription factor
2541.m00041	Zn_2Cys_6 transcription factor
2541.m00034	NPP1 domain protein

Tab. 5 - Genes of the cluster of particular interest

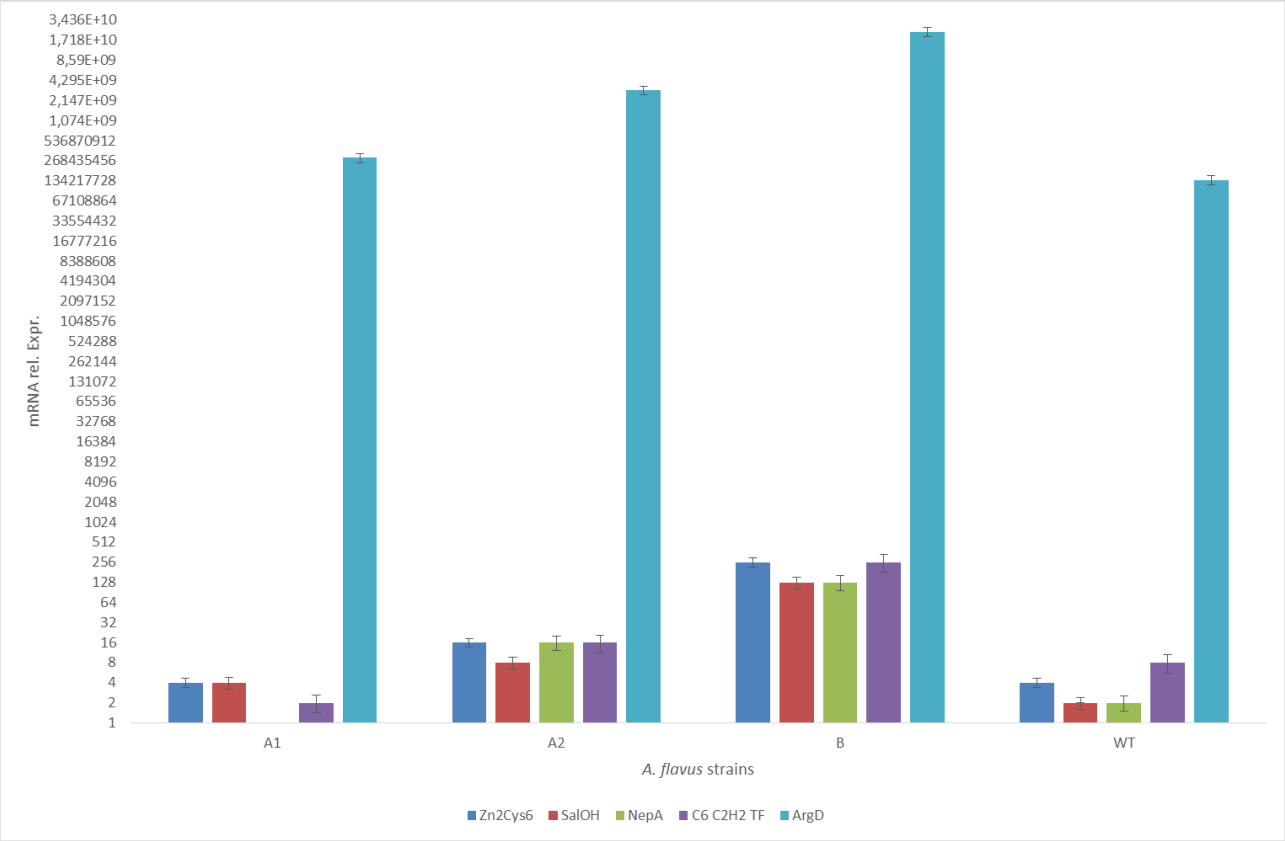


Fig. 46 - Zn2Cys6, Sal-OH, NepA, C6 and C2H2 TF, *arg*D gene expression

This approach should confirm AFLA_096370 deletion as well as its involvement in the regulation of expression of genes inside cluster *32*. In fact, the peculiarity of this cluster, is represented by the presence of another transcription factor whose role can synergize or

antagonize AFLA_096370, as verified in other SMs clusters as the aflatoxin gene cluster (consider AflJ and AflR for comparison; Ehrlich *et al*., 2012).

Regarding information related to the expression profile, data collected from RT-qPCR are, instead, very different: all Knock-out mutants displayed Zn_2Cys_6 gene expression and a very similar relative expression values for the other genes analysed; on the other hand, the overexpression mutant showed a higher level of expression for all the genes considered.

Results shown by qRT-PCR analysis revealed in a rather unequivocal way that Zn_2Cys_6 and other genes whose expression is related to this transcription factor, is expressed in WT, AFC-1 and all mutants analyzed above gene is expressed in all samples except in AFC-1 (where it was interrupted)

In the light of these, we can hypothesize that, probably, the AFLA_096370 deletion cassette has been inserted ectopically (see selective PCR results) and not targeted in a site-specific way the *native* AFLA_096370 locus. This cassette, in fact, contains the *argD* gene, which allow the AFC-1 auxotroph to growth without arginine together with the 5'- and 3'UTR of AFLA_096370. It is known that the presence of transgene containing parts of a *naïve* gene may interfere with the expression of the naïve gene by activating the RNAi cell machinery (Dang *et al.*, 2011). In relation to this, the alteration of phenotype observed (see figures above and physiological results below) may be partly explained by the interference exerted by the expression of our deletion cassette.

Perhaps, probably because of this ectopic insertion, the knock-out mutants have survived only for a few generations. The B mutant, on the other hand, went ahead and was used for subsequent experiments. Due to the increased level of expression of the genes examined, this mutant was renamed C32 OE.

4. CHARACTERIZATION OF INOCULATED KERNELS

4.1 RT-qPCR

To clear up the results, we performed the cDNA amplification of some gene inside the cluster 32 to investigate the expression levels during host infection. According to our hypothesis on cluster 32, and its particular relevance for pathogenesis process, we focused on three genes inside the cluster, Sal-OH, Nep A and Quercetin Deoxygenase.

Gene ID	Description
AFLA_096290	**Salicylate hydroxylase, putative**
AFLA_096430	**NPP1 domain protein**
AFLA_096260	**Aromatic ring-opening dioxygenase family protein**

The expressing levels of Sal-OH followed an increasing trend with the progress of the days and the infection itself. These results lead to think the importance of this gene in the onset and progression of infection in host plant. These graphics show also the evolution of expressing profiles of NepA, necrosis- and ethylene- inducing protein, in the studied strain. It can be noticed how the expression of necrosis factor start from the second day and increasing slightly over time. Finally, the expression of quercetin deoxygenase, an enzyme able to inactivate some flavonoids produced by the plant to defend itself, was also analyzed.

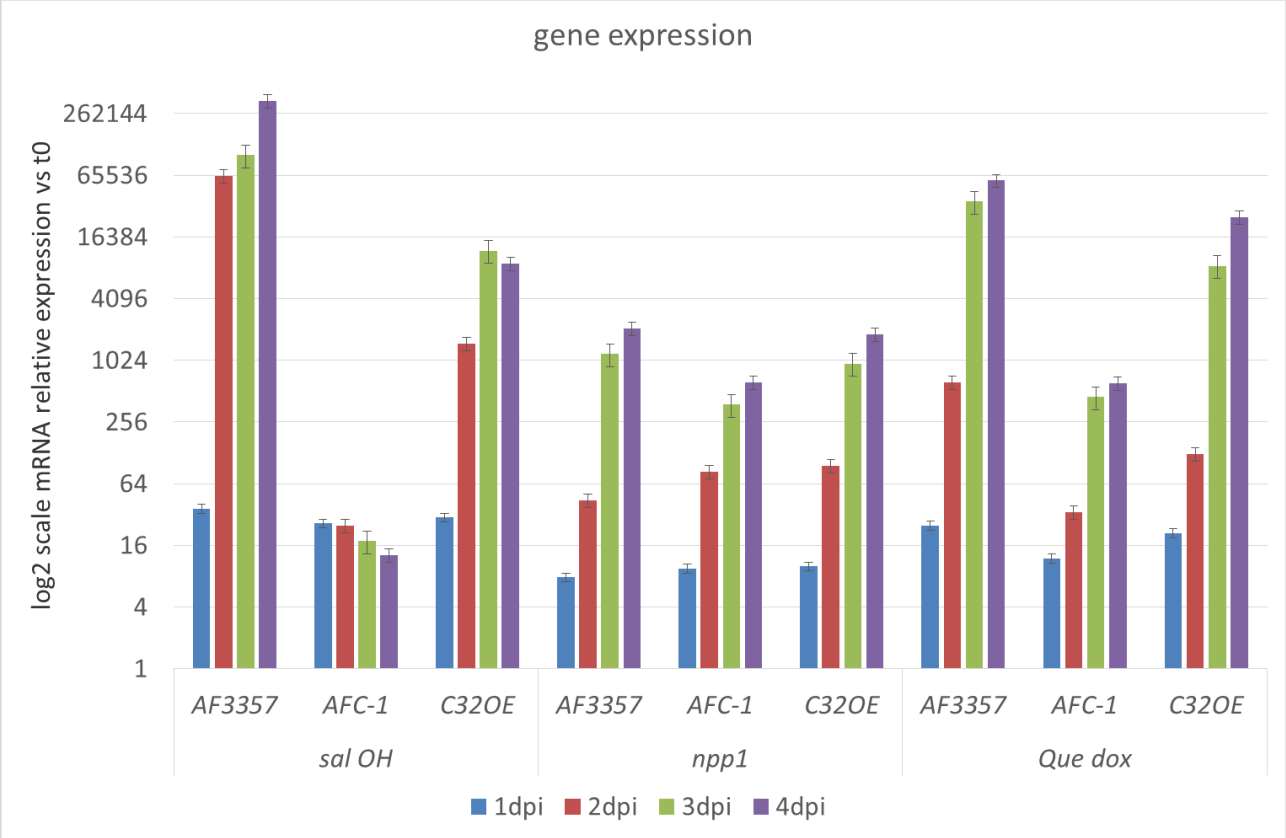
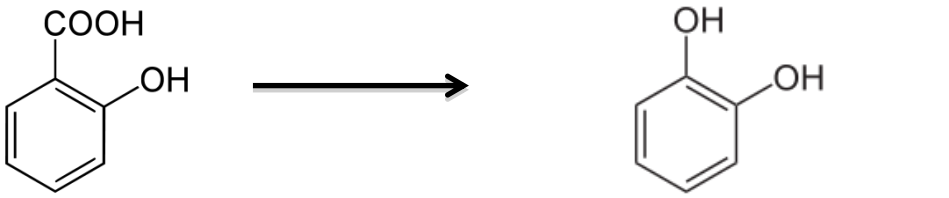


Fig. 47 - Expression levels for Sal-OH, npp1 and Quercetin Deoxygenase during the infection process, from T 24 hours to T 4 days, in the different strains. Each results represent the media ± DS of three technical replicates.

4.2 Salicylic acid and Jasmonic acid analysis

To verify, the efficacy of Sal-OH activity into maize caryopses infected with Wt strain we perform a LC-MS/MS (MRM) monitoring of salicylic acid and of its by-product catechol (Fig. 48).



Salicylic acid Catechol

Fig. 48 Mechanism of action of salicylate hydroxylase

Plants have evolved a number of inducible defense mechanism to respond to both biotic and abiotic stress. Systemic Acquired Resistance (SAR) produced by pathogen attack is based on salicylic acid (SA) signalling and leads to pathogenesis-related proteins (PR) and phytoalexin synthesis, which may confer protection against latter attacks. A similar response is produced when the plant is attacked by a necrotrophic pathogen.

The SA is implicated in the SAR and with this mechanism the plant can defend itself from pathogen attack, but *A.flavus* have the salicylate hydroxylase to induce disease without being stop.

This is demonstrated in the reaction products, obtain from the analyses of inoculated kernels, where the cathecol presence increases during the time lapse and the SA decreases. It is evident that *A.flavus* salicylate hydroxylase is necessary to arrest SA, through degradation, in particular from the aleuronic layer. The inhibition of maize SA is one of the way, which pathogens have, to stop the defence responses activated from planta under attack.

As for jasmonic acid, no significant differences were found (data not showed)

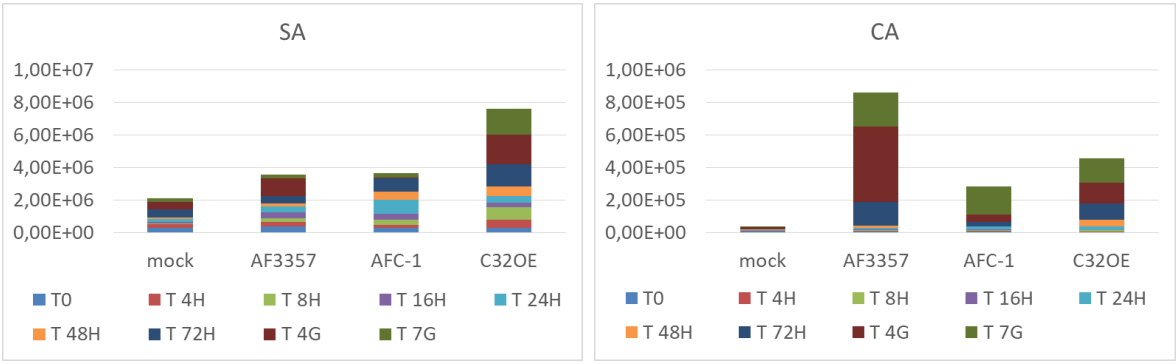


Fig. 49 - Infection triggers SA biosynthesis (defence genes & PCD). A. *flavus* converts part of SA in CA (~10%)

4.3 Histology protocol

We analyzed three different strains: WT, AFC-1 and C32 OE. The first analysis performed was the histological assay with different withdrawals at 2, 3 and 4 days. The maize kernels were inoculated with a pin bar wet with a solution of fungi spores (1 x 10^6 spores/mL).

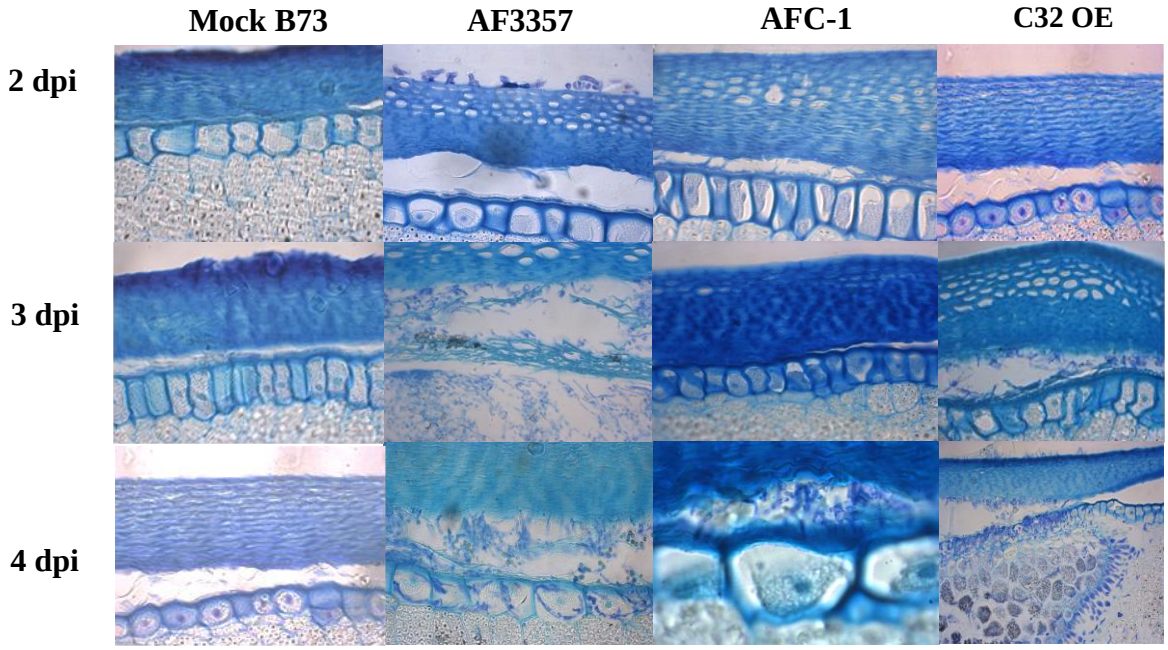


Fig. 50 - Histological assay for WT, AFC-1 and Mutant C32 OE

At 2 days, on the pericarp of AFC-1 and mutant C32 OE infected kernels, *A. Flavus* conidia are absent even if it is visible a PCD initial event.

At 3 days from infection, in mutant C32 OE, the pericarp and the aleuronic layer are both infected, but not the embryo. Instead, AFC-1 is similar at the T 2 days.

At 4 days for AFC-1 and mutant C32 OE are similar with the fungus presence between the pericarp and the aleuronic layer. However, even in this case, the aleuronic layer could represent a barrier to the infection during the early stages, but with the disease progression the kernels eventually collapse (at 7 days after infection), independently from the fungal strains.

4.4 GUS histochemical assay

In order to follow the progression of the fungus in the caryopsis, another type of experiment was performed using the GUS histochemical assay with two strains: NepA KO and NepA OE. This two strains are the first one the kock-out for NepA gene (Necrosis Ethylene-inducing Peptide) and the second one is the overexoressing strain for NepA gene.

The maize kernels was infected with a solution of 1x 10^6 spores/mL each with a pin bar directly inside the kernels. Exploiting the reporter gene presence, we followed *A.flavus* pathogenic development in kernels for 7 days. The time lapse of withdrawals is T 4h, T 8h, T 16h, T 24h, T 48h, T 72h, T 4d, and T 7d.

Data not shown for the early times as T4h and T8h because the *A.flavus* growth was not detectable.

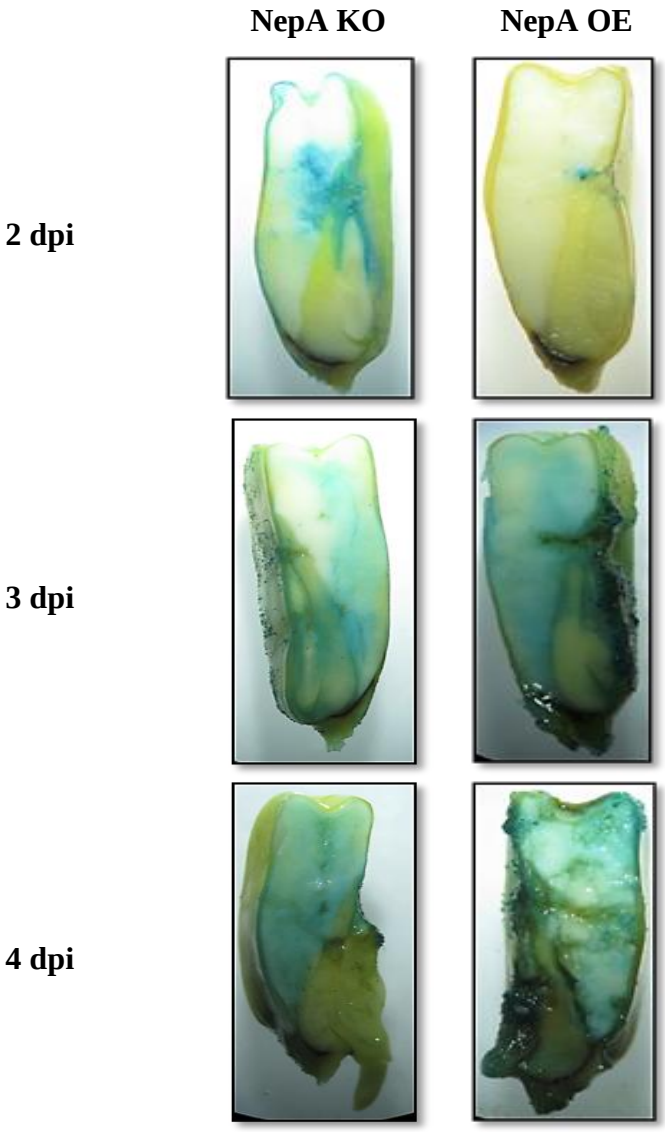


Fig. 51 - GUS histochemical assay for NepA KO and Nep A OE

During the early stage of infection development, it's remarkable the barrier effect causing by the aleuronic layer. With the going ahead of the days, *A.flavus* is capable of establishment inside the endosperm without being so aggressive against the embryo. In particular, in fig.51 for NepA KO at T 96 H is visible the germination as symbol of a living embryo which seeks an escape route from the fungus attack. However, the kernels are destined to death after 7 days.

The similar behaviour of the knock-out strain and the over-expressing for NepA, a necrosis factor, is probably ascribable to a functional redundancy that allows a correct infection development for NepA KO and increased activity so as to equate the entire pathogenicity cluster for NepA OE. It is suggested here that a major role in defining the pathogenic strategy in this particular situation, i.e. a wounded kernel, has to be ascribed to the effector salicylate hydroxylase, produced by this fungus.

5. RUTIN DEGRADATION

The analyzes were carried out on the culture medium in which *aspergillus flavus* (starting concentration 1 x 10^6 spores/mL) was grown for 7 days.

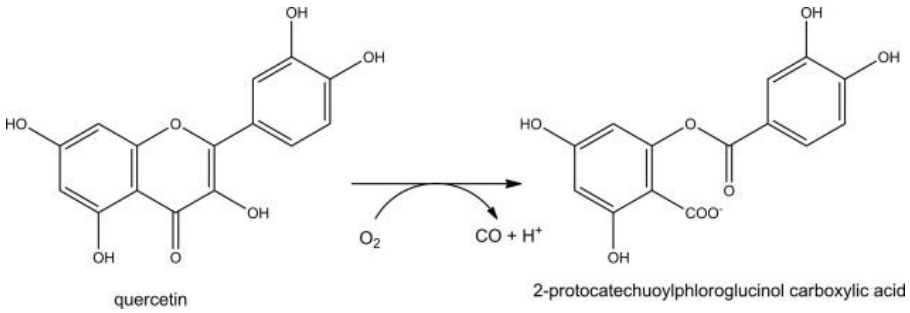


Fig. 52 - Mechanism of action of Quercetin Dioxygenase

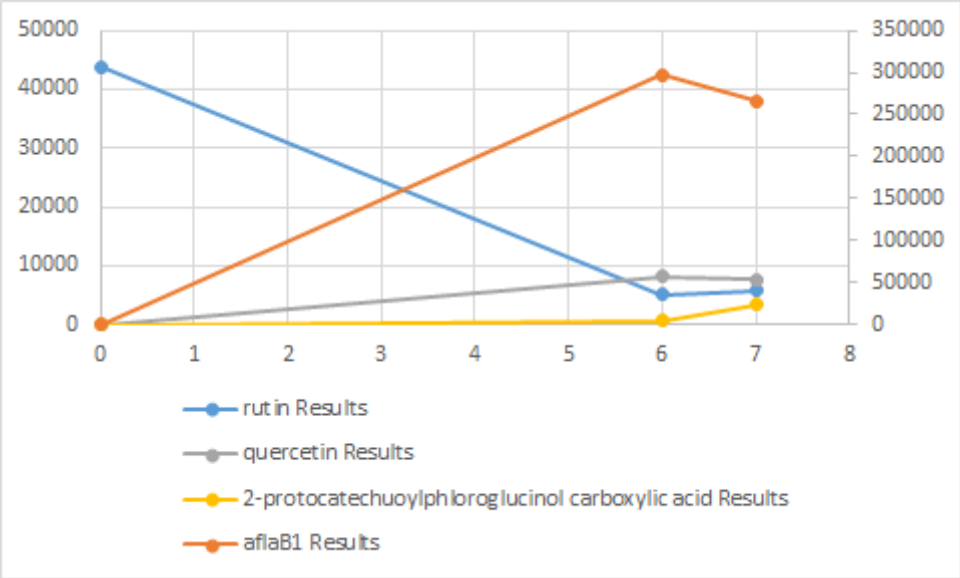


Fig. 53 - Rutin degradation and Aflatoxin production in *A. flavus* WT

A. flavus may degrade rutin as well as quercetin (maize phenolics)

The first analysis performed was the histological assay with different withdrawals at 2, 3 and 4 days. The maize kernels were inoculated with a pin bar wet with a solution of fungi spores (1×10^6 spores/mL).

CONCLUSIONS AND FUTURE PROSPECTIVES

A.flavus is a saprophytic filamentous fungus that is distributed all over the world. It can produced an abundance of secondary metabolites and the most studied is aflatoxins which contaminate crops pre- and post harvest and cause animal and human diseases.

It is known that *A.flavus* causes necrosis on infected plants, but in our study we find some interesting structures, one is shown below. These imagine is an appressorium probably indicating a biotrophic phase and then a necrotrophic phase during its infection on *Zea mays*.

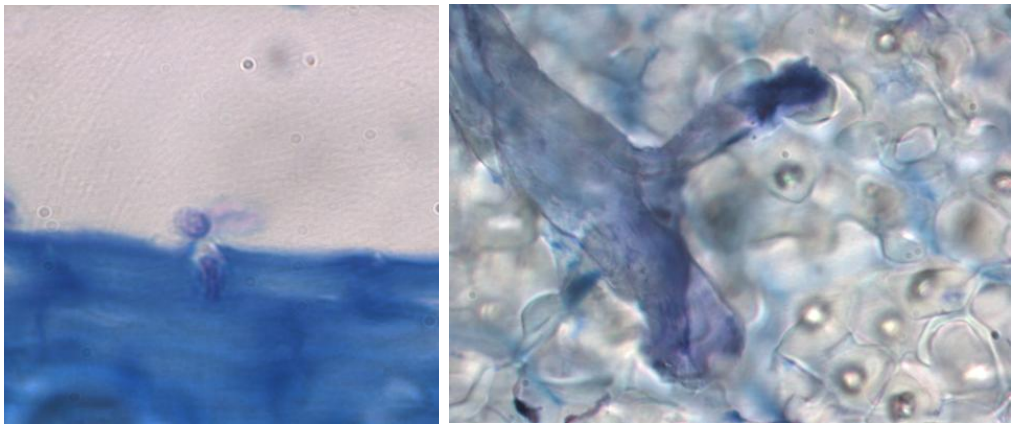


Fig. 54 - Appressorium of *A. flavus* on kernel of *Zea mays*

Within this frame, several mutants of A. flavus impaired or enhanced in specific functions (e.g. cluster 32 overexpression, NepA KO and OE strains) were checked for their ability to cause disease in maize caryopses. A scenario emerged in which fungal progression through living tissues (e.g. aleuron) is accompanied by a significant rise in the level of fungal effectors, such as SalOH and NepA, and by a degradation of SA that, in turn, appears strategic for the fungus to bypass caryopses defences and attenuate programmed cell death phenomena naturally occurring in the aleurone layer of maturating kernels (Perfect and Green, 2001; Reverberi et al., 2013; Fountain et al. 2014; Glazebrook 2005; Vargas et al., 2012).

This is demonstrated from the results of qRT-PCR and the HPLC-MS/MS, which support the hypothesis of the importance of salicylate hydroxylase in the degradation of SA to stop the plant defenses.

Genes within cluster 32 (subgroup 1 and nep A group) are expressed soon after kernel infection; it is therefore essential to suppress the transcription factor.

As regards our experiments cycles, it's plausible to assume that the integration of different constructs did happen into the *A.flavus* genome but the PCR data show that the integration

was occurred ectopically, without the homologous recombination with *AFLA_096370*, Zn_2Cys_6. From the collected data, our goal is to continue to the transformation process with pyrithiamine, the best selection marker among those tested.

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