FUNGAL PHYTOTOXINS; STORY-CASE OF CASSIICOLIN, A NEW PROTEINACEOUS HOST-SELECTIVE TOXIN FROM *Corynespora cassiicola.*

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Toxins: for the worst and the best

Toxins are poisons produced by natural organisms that disturb the normal functioning of other organisms, by altering specific target molecules usually at low concentrations. Bacteria, viruses, fungi, algae, plants or animals, most genera include species able to produce toxins, which generally confer a physiological advantage to the organisms that produce them. The scientists' interest for natural poisons has largely increased for the past 4 decades for two reasons: On one hand, toxins can be a threat for human kind, through natural intoxication or biological weapons. A better knowledge of these poisons soon appeared necessary in order to develop methods for detection or detoxification. On the other hand, some of these active molecules proved very useful, when properly used, as medicinal substances (1, 2). Among the first toxins to be discovered, in 1888-1990, were three bacterial toxins responsible for severe pathologies: diphtheria, tetanus and botulism respectively (3). Because these toxins, as well as most bacterial toxins identified so far, were small modified peptides, the word "toxin" in bacteriology was restricted to the biological poisons of proteinaceous nature. In other fields of biology, where toxins are mainly low molecular weight compounds with a wide range of chemical structure, the word "toxin" refers to any toxic organic molecule of biological origin.

Fungal toxins

Researches on secondary metabolites produced by filamentous fungi were developed with the objective to discover useful bioactive substances such as antibiotics or herbicides but also to face the problem of parasitism in plants (4). Some toxin-producing fungi developing on plants or plant-derived products may represent a serious threat as contaminants of the food chain, causing intoxication in humans or animals. The word "mycotoxins" describes secondary metabolites of low molecular weight, toxic for

vertebrates, and produced by filamentous micromycetes (which excludes toxins from macromycetes). So far, more than 300 mycotoxins have been listed, from which only a dozen, belonging to 3 main genera (*Aspergillus, Penicillium and Fusarium*), are regularly involved in human or animal intoxications (Table 1). Whether or not the mycotoxins produced by plant-hosted fungi are also determinants of pathogenicity in the host plant is not always very clear (5). However, progresses in the cloning of genes involved in the mycotoxins biosynthesis pathways, together with techniques of gene disruption, should allow demonstrating the role of each toxin in phytotoxicity.

Table 1: The main mycotoxins. Review (5). Ph.: phytotoxicity

Toxin	Fungus	Chemical structure	Ph
Trichothecenes (T-2 toxin, nivalenol, deoxinivalenol)	*Fusarium spp*	Cyclic sesquiterpenes	Yes
Fumonisins (B1, B2, B3, B4)	*Fusarium moniliforme*	Amino polyalcohols.	Yes
Aflatoxines (B1, G1)	*A.s flavus, A. parasiticus*	Polyketides (Bisfuranocoumarins)	Yes
Ergot alkaloids (Clavine, ergotamine)	*Claviceps purpurea*	Alkaloids derived from amino acids and isoprenoids precursors	?
Zearalenones	*Fusarium graminearum.*	Poliketides	?
Ochratoxines	*Aspergillus and penicillium spp.*	Chlorinated cyclic pentaketides	?
Eniatins	*Fusarium spp*	Cyclic depsipeptides	Yes
Beauvericins	*Fusarium spp*	Cyclic depsipeptides	?
Moniliformin	*Fusarium spp*	Cyclobutane derivative	Yes
Patuline (clavacine)	*Aspergillus and Penicilium spp.*	Cyclic tetraketide	Yes

Phytotoxins can be divided into two categories, based on selectivity (= specificity): non-host-selective and host-selective toxins. Non-host-selective toxins are involved in a broad range of plant-fungus interactions, and will therefore not usually determine the host range of the pathogen producing them. Although they generally act as virulence factors and intensify the symptoms severity, they are not absolutely required for establishing the disease (6). Host-selective toxins (HSTs), are both determinants of pathogenicity (the purified toxin is able to induce the disease) and host-range (the purified toxins share the same host range as the organisms that produce them). Several reviews on HSTs are available (7-9). In many pathosystems, disease occurs only in the absence of either the pathogen avirulence gene or the corresponding plant resistance gene, following the famous gene-for-gene theory. The product of the avirulence gene is referred to as “agent of

incompatibility". In contrast, in the case of HST-producing pathogens, the toxin can be referred to as "virulence factor or "agent of compatibility" in the sense that it triggers pathogenicity by inducing host cell death, for the physiological benefit of the pathogens (7, 9). Host cell death therefore is a common feature to both interaction types, but while it occurs as a resistance mechanism in one case (hyper-sensitive reactions), it is causal of the disease in the other case.

Most phytopathogenic fungal toxins are low-molecular weight secondary metabolites of widely diverse chemical structures, including cyclic peptides, terpenoids, polyketides,…(Tables I and II). They are assembled by large enzymatic complexes encoded by co-regulated clustered genes, including non ribosomal synthetase or polyketide synthase. In rare cases, HSTs can be proteins encoded through classical ribosomal synthesis. Their mode of action can be as varied as their chemical structure, although it remains still largely unknown in most cases.

Table 2: Fungal host-selective toxins. Reviews (9, 10).

Toxin	Fungus (pathotype)	Chemical structure	Ref.
Victorin	*Cochliobolus victoriae*	Cyclic pentapeptide (halogenated)	(9)
T-Toxins	*Cochliobolus heterostrophus*	Linear polyketols	(9)
HC-Toxins	*Cochliobolus carbonum*	Cyclic tetrapeptides	(9)
HS-toxins	*Bipolaris sacchari*	Glycosylated sesquiterpenes	(9)
AAL-toxin	*Alternaria alternata* (tomato)	Aminopentol esters	(10)
AK-toxin	*A. alternata* (japanese pear)	Epoxy-decatrienoic esters	(10)
ACT-toxin	*A. alternate* (tangerine)	Epoxy-decatrienoic esters	(10)
AF-toxin	*A. alternata* (strawberry)	Epoxy-decatrienoic esters	(10)
ACTG-toxin	*A. alternata* (tangerine)	Terpenoid	(10)
ACRL-toxin	*A. alternata* (rough lemon)	Terpenoid	(10)
AM-toxin	*A. alternata* (apple)	Cyclic tetrapeptide	(10)
Maculosin	*A. alternate* (spotted knapweed)	Diketopiperazine cyclo(-L-Pro-L-Tyr-)	(10)
AS-toxin	*A. alternata* (sunflower)	Peptide	(10)
Destruxin B	*Alternaria brassicae*	Cyclic hexadepsipeptide	(10)
PM-toxin	*Phyllosticta maydis*	Linear polyketols	(9)
Peritoxins (A, B)	*Periconia circinata*	Cyclized peptide /chlorinated polyketide	(9)
SV-toxin	*Stemphylium versicarium*	Partially characterized	(11)
Ptr-Tox A	*Pyrenophora tritici-repentis*	13,2 kDa protein	(9, 12)
Ptr-Tox B	*Pyrenophora tritici-repentis*	6.6 kDa protein	(9, 13)
Cassiicolin	*Corynespora cassiicola*	2.9 kDa polypeptide	(14, 15)

Cassiicolin, a new protein host-selective toxin, causal agent of the *Corynespora* leaf fall disease in rubber tree.

Corynespora cassiicola is a phytopathogenic ascomycete with a wide host range (390 records for *Corynespora cassiicola* in the SBML Fungus-Host Distributions database), including economically important crops such as rubber tree, tomato, cucumber, cotton, soybean, tobacco, cocoa…. It generates necrotic lesions on most organs of target species, affecting growth, yield, and in severe cases leading to the death of the plant.

In rubber tree, *Corynespora* Leaf Fall Disease (CLFD) is characterized by necrotic lesions developing along the leaf veins and giving a typical "fish-bone" appearance (16). Both young and mature leaves can be affected, leading to massive defoliation. First reported in India and Malaysia, CLF disease has sprayed rapidly since and is now a major problem in most Asian and African rubber producing areas where it accounts for up to 25% of yield loss. Although many plants can be affected by *C. cassiicola*, each isolate has a specific range of host species, and even a specific range of host genotypes within species. Various strains of *Corynespora* display symptoms of various severity depending on the host genotype, going from HR("hypersensitive response")-like responses in resistant interactions, with a very limited necrotic spot at the infection site, to the development of water-soaked necrosis through the whole leave. Tolerant interactions display intermediate symptoms.

It soon appeared that a toxic substance was produced by the fungus, since crude filtrates from *C. cassiicola* cultures were able to induce disease symptoms. We have purified this toxin, named cassiicolin, from the supernatant of a *C. cassiicola* culture (strain CCP, isolated from rubber tree in Philippines). Our optimized protocol involved one step of reverse phase chromatography on GE Healthcare Source 15 RPC columns, followed by size exclusion chromatography on a GE Healthcare Superdex 30 Prep-Grade column (14). The toxicity of the fractions was monitored on detached rubber tree leaves of the sensitive genotype PB260. Exogenous application of purified toxin and fungal inoculation induced identical cellular damages in rubber tree leaves, including plasmolysis and chloroplast alterations. Cassiicolin behaves like a typical host-selective toxin, sharing the same host range as the fungus strain it originates from (15, 17).

The structure of cassiicolin was determined by mass spectrometry, amino acid sequencing (Edman) and NMR spectroscopy (14, 15). Cassiicolin is a small peptide of 27 amino acids, with 6 cysteins involved in 3 disulfide bounds and 2 post-translationally

modified residues: an N-terminal pyroglutamatic acid and a threonine with a single methyl-mannose in the second position. Cassiicolin shares no sequence homology with any previously described peptide or protein. Its over-all fold consists of three strands arranged in a right-handed twisted, antiparallel ß-sheet knitted by the three disulfide bonds. Comparative analysis of the 3D structure, using the FAT-CAT server (http://fatcat.burnham.org/), revealed a similar fold within a family of small trypsin-like protease inhibitors isolated from grasshoppers (18). However, cassiicolin shares no sequence homology with these protease inhibitors and lacks their characteristic substrate binding loop. Probably, this simple, compact, and well organized structural motif represents one of the few highly stabilized "minimal" scaffold – with a high sequence permissiveness – that nature has selected to evolve over different phylla and to support different functions. So far, the action mechanism of cassiicolin remains unknown.

Among the fungal Host-Selective Toxins (HSTs) characterized so far, very few are proteins (Table 2). The first and best characterized protein HST is Ptr ToxA, produced by *Pyrenophora tritici-repentis*, causal agent of Tan (or yellow) spot in wheat (19-21). It is encoded by a single gene (22). Ptr toxA is imported into toxA-sensitive but not – insensitive mesophyll cells, probably following a mechanism similar to the vitronectin-integrin internalization system in mammalian cells (12, Manning, 2005 #1873). Once internalized, Ptr ToxA interacts with a chloroplast-localized protein (23). Virulence of the fungus requires both the production of Ptr ToxA and the presence in the wheat genome of a dominant susceptibility gene, *Tsn1* (24, 25). Sensitivity to the purified toxin collocates to the same genetic locus as susceptibility to the disease. Recently, a gene sharing 99.7% similarity with *Ptr ToxA* was discovered in the genome of *Stagonospora nodurum*, another major wheat pathogen. *Sn ToxA*, *like Ptr ToxA*, interacts with the sensitivity locus Tsn1 in wheat. The *ToxA* gene from *S. nodurum* may have been transferred to *P.tritici-repentis* by horizontal gene transfer (26).

Ptr ToxB, an other HST produced by *P. tritici-repentis*, is also a protein, encoded by a multi-copy gene, *ToxB* (13, 27, 28). Interestingly, a related gene, *toxb*, was identified from a non pathogenic isolate. Until then, HST genes had been found exclusively in pathogenic isolates with no corresponding allele or locus in non pathogenic isolates. *ToxB* and *toxb* genes differ in their transcriptional regulation

Although *Pyrenophora tritici-repentis* represents an interesting model as the first described phytopathogenic fungus producing proteinaceous HSTs, it does not provide

clues about the putative function of cassiicolin, which is structurally unrelated to Ptr toxins.

Conclusion

Cassiicolin is a new host-selective toxic protein, sharing no homology with any previously described protein, which suggests a new mechanism of aggression in plants. The structural characterization of cassiicolin opens the way to cloning of the corresponding gene. The analysis of its diversity and/or differential regulation among various isolates, together with the search for the target molecule in the host plant, will help understanding the interaction mechanisms and host-selectivity of the toxin. Another question to address will be the importance of the sugar moiety for toxicity. Post-translational modifications are known to play a key role in the activity of some toxic molecules. As an example, deglycosylation of a phytotoxin associated with sheath blight disease in rice, *via* an extracellular α-glucosidase from *Trichoderma viride*, resulted in the inactivation of the toxin (29). The structural characterization of cassiicolin also provides a new basal motif that could be used for the design of analogue proteins with novel functions, including antagonists.

References

1. Ménez A, Gillet D, Grishin E. 2006. In *Recent Research Developments in Toxins from Bacteria and Other Organisms*, ed. D Gillet, J Ludger, pp. 1-33. Trivandrum-695 023, Kerala, India: Research Signpost
2. Ménez A, Goyffon M, Gillet D. 2006. *Biofutur* 272: 21-42
3. Alouf JE, Popoff MR. 2006. *The comprehensive Source Book of Bacterial Protein Toxins*. London: Academic Press
4. Grovel O, Kornprobst J-M, Pouchus Y. 2006. *Biofutur* 272: 39-42
5. Desjardins AE, Hohn TM. 1997. *Mol. Plant Microbe Interact.* 10: 147-52
6. Ballio A. 1991. *Experientia* 47: 783-90
7. Walton JD. 1996. *Plant Cell* 8: 1723-33
8. Markham JE, Hille J. 2001. *Mol. Plant Pathol.* 2: 229-39
9. Wolpert TJ, Dunkle LD, Ciuffetti LM. 2002. *Annu Rev Phytopathol* 40: 251-85
10. Thomma BPHJ. 2003. *Mol Plant Pathol.* 4: 225-36
11. Singh R, Bugiani P, Cavanni H, Nakajima M, Kodama H, et al. 1999. *Phytopathology* 89: 947-53
12. Sarma GN, Manning VA, Ciuffetti LM, Karplus PA. 2005. *Plant Cell* 17: 3190-202
13. Martinez JP, Oesch NW, Ciuffetti LM. 2004. *Mol. Plant Microbe Interact.* 17: 467-74
14. de Lamotte F, Duviau MP, Sanier C, Thai R, Poncet J, et al. 2007. *J Chromatogr B Analyt Technol Biomed Life Sci* 849: 357-62

15. Barthe P, Pujade-Renaud V, Breton F, Gargani D, Thai R, et al. 2007. *J Mol Biol* 367: 89-101
16. Ramakrishnan T, Pillay P. 1961. *Rubber Board Bulletin* 5: 52-3
17. Breton F, Sanier C, d'Auzac J. 2000. *J. Rubber Res.* 3: 115-28
18. Malik Z, Amir S, Pal G, Buzas Z, Varallyay E, et al. 1999. *Biochim Biophys Acta* 1434: 143-50
19. Ballance GM, Lamari L, Bernier CC. 1989. *Physiol. Mol. Plant Pathol.* 35: 203-13
20. Tomas A, Feng G, Reech G, Bockus W, Leach J. 1990. *Mol. Plant Microbe Interact.* 3: 221-4
21. Zhang H-F, francl L, Jordahl J, Meinhardt S. 1997. *Phytopathology* 87: 154-60
22. Ciuffetti LM, Tuori RP, Gaventa JM. 1997. *Plant Cell* 9: 135-44
23. Manning VA, Hardison LK, Ciuffetti LM. 2007. *Mol Plant Microbe Interact* 20: 168-77
24. Faris JD, Anderson JA, Francl LJ, Jordahl JG. 1996. *Phytopathology* 86: 459-63
25. Stock WS, Brûlé-Babel AL, Penner GA. 1996. *Genome* 39: 598-604
26. Friesen TL, Stukenbrock EH, Liu Z, Meinhardt S, Ling H, et al. 2006. *Nat Genet* 38: 953-6
27. Strelkov SE, Lamari L, Ballance G. 1999. *Mol. Plant Microbe Interact.* 12: 728-32
28. Martinez JP, Ottum SA, Ali S, Franci LJ, Ciuffetti LM. 2001. *Mol. Plant Microbe Interact.* 14: 675-7
29. Shanmugam V, Sriram S, Babu S, Nandakumar R, Raguchander T, et al. 2001. *J. Appl. Microbiol.* 90: 320-9