Multi enzyme systems involved in astin biosynthesis and their use in heterologous astin production

Project acronym: MESIAB
Project no: EIB.10.004

ERA IB
European Research Area
Industrial Biotechnology

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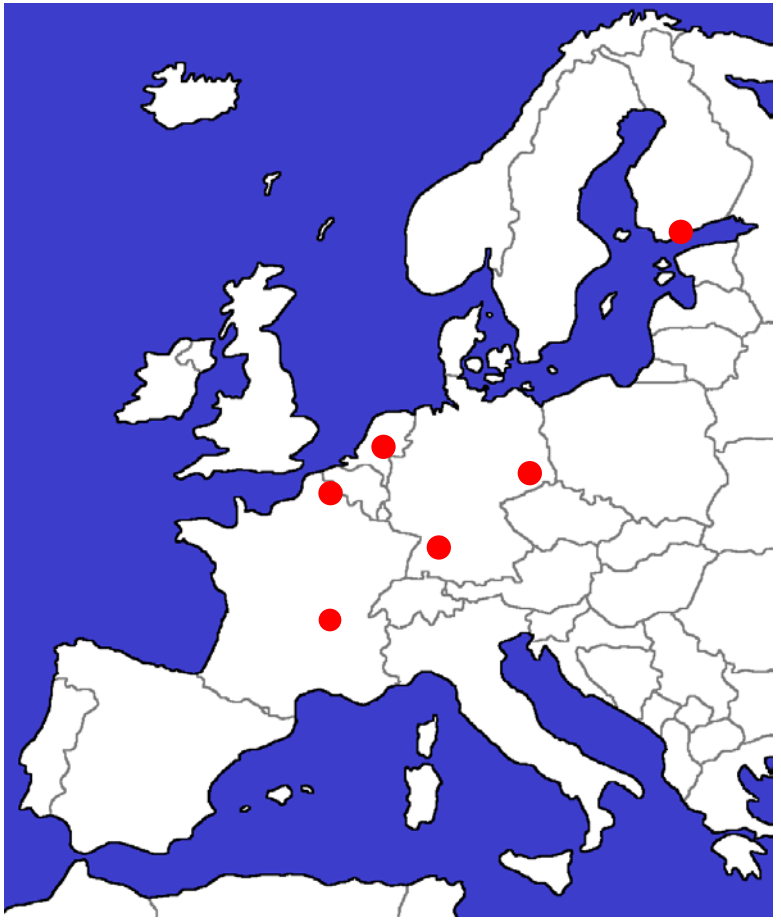
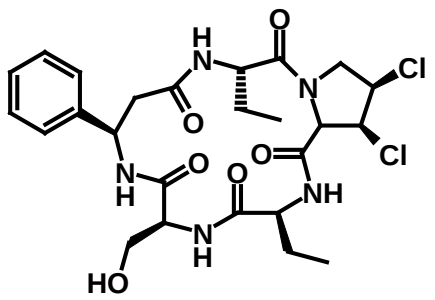


Fig. 1: http://www.digitale-europakarte.de/europakarte.png

Project aim

Enhancing the production of astins using molecular genetic tools and screening for new biological activities and novel applications of astins



Chemical structure of astin C

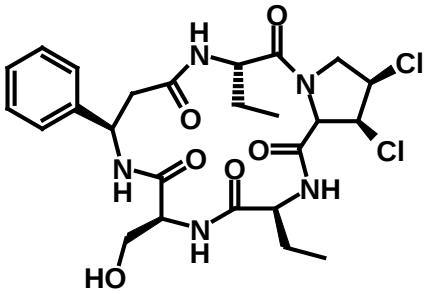
General project approach

Detection, cloning and heterologous expression of the individual genes involved in astin biosynthesis

Combining the genes into a cluster, expression of the cluster in Streptomyces, yeast and hairy root cultures

Screening for new biological activities and novel applications for astins using cell cultures and transcriptomic technologies

Detection of astins in *Aster tataricus*



Chemical structure of astin C

Dried roots of *Aster tataricus*

LC-MS detection methods were established for astins

- We have detected astins from dried root samples of *Aster tataricus*
- These are mainly dichlorinated, but some do not have the same mass as published astins

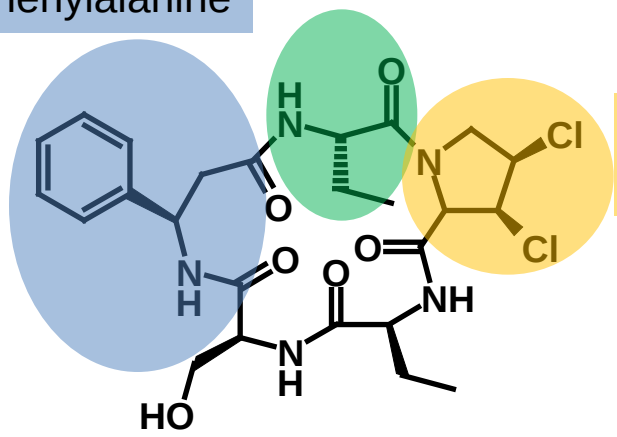
Cyclochlorotine from *Penicillium islandicum*

Penicillium islandicum

β-Amino phenylalanine

Amino-butyric acid

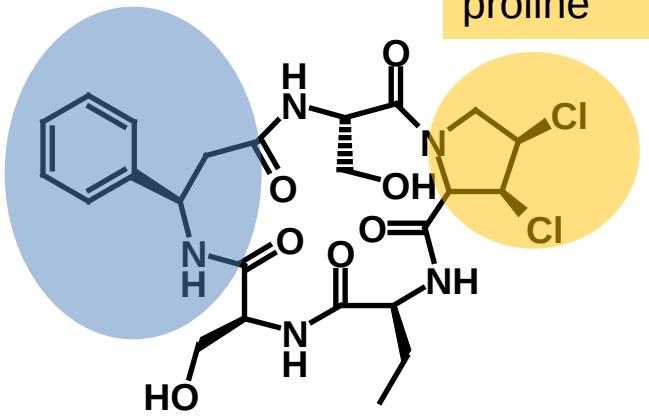
Dichloro-proline



Astin C

β-Amino phenylalanine

Dichloro-proline



Cyclochlorotine

Detection of astin / cyclochlorotine biosynthetic genes

- **Both biosynthetic pathways can be assumed to require highly similar enzymes**
- **Working with *P. islandicum* should be faster and easier**
- **Thus, it was decided to sequence the *P. islandicum* genome and use the detected genes for searching for the corresponding genes in *A. tataricus***

P. islandicum sequence analysis

→ **Candidate genes are present, but <u>not</u> clustered**

Genome analysis

Macrocyclisation of NRPS

Bacteria

Du and Lou, 2009, Nat Prod Rep

Te

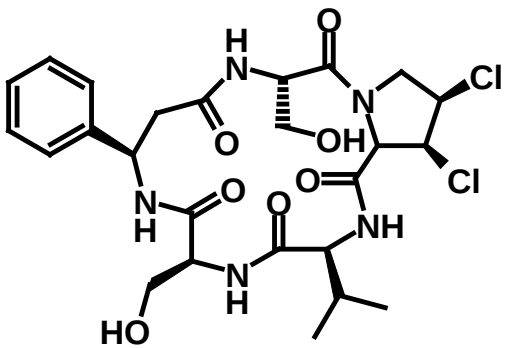
Fungi

Gao et al., 2012, Nature ChemBiol

CT

Literature data show that a terminal condensation (CT) domain is required for macrocyclisation of cyclic nonribosomal peptides in fungi

This information means that the NRPS involved in cyclochlorotine production will most likely contain a CT domain



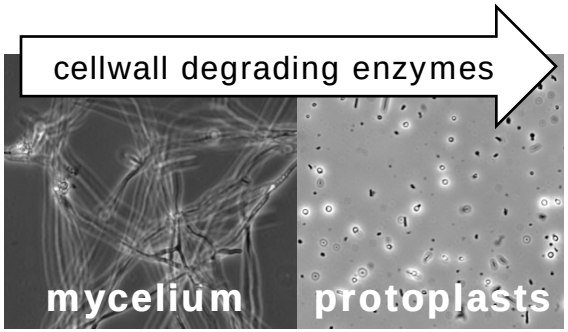
Cyclochlorotine

Specific signature

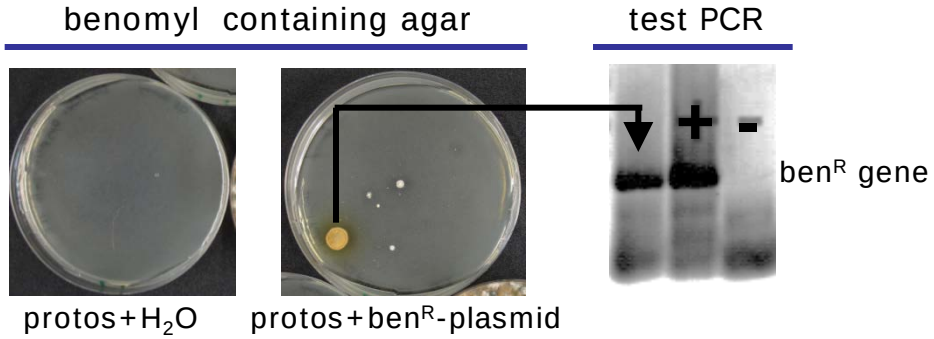
Present, ending the NRPS?

Genetic engineering of *P. islandicum*

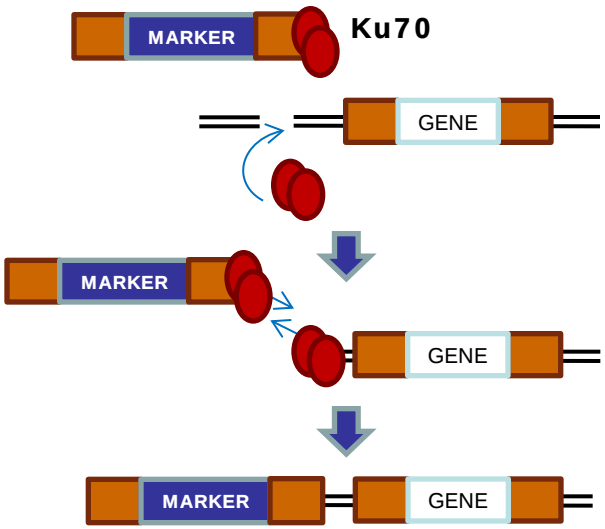
1 Protoplasting successful



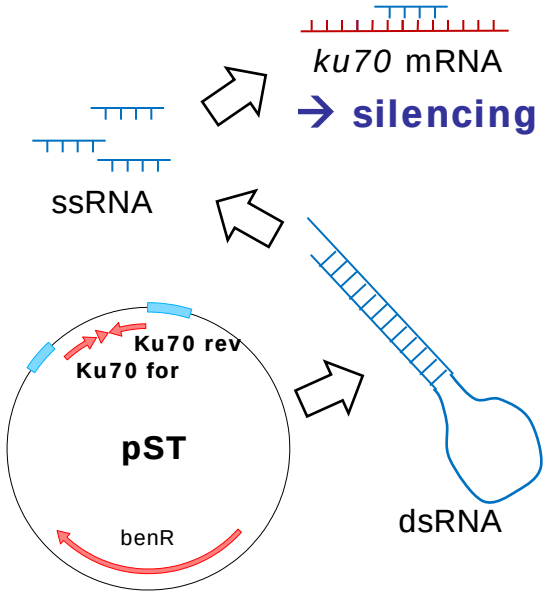
2 Protoplast-transformation successful

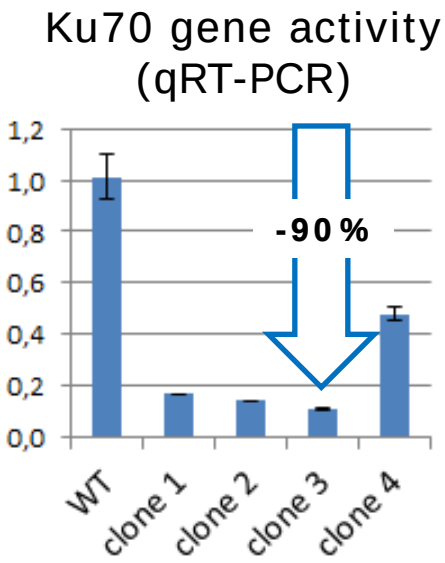


3 NHEJ deficient strain established

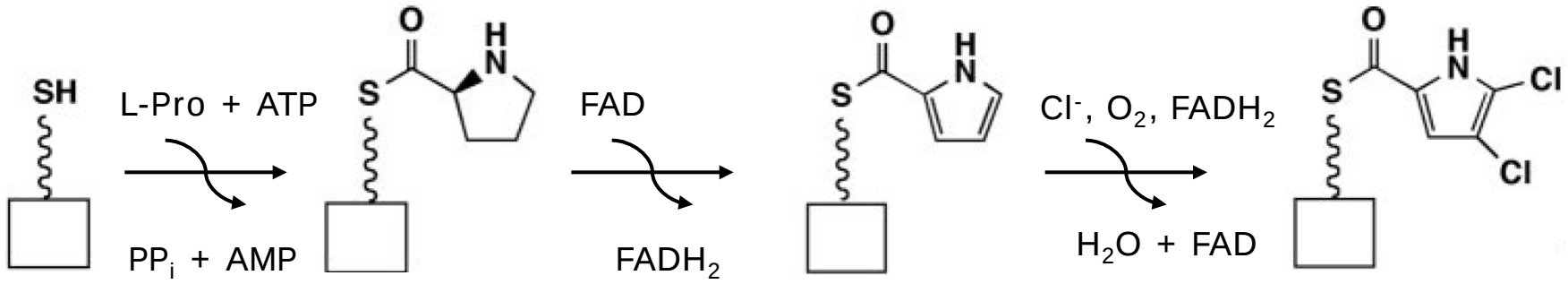


non-homologous end joining (NHEJ)





Prolyl dehydrogenase (PRODH)



peptidyl carrier protein

proline adenyltransferase

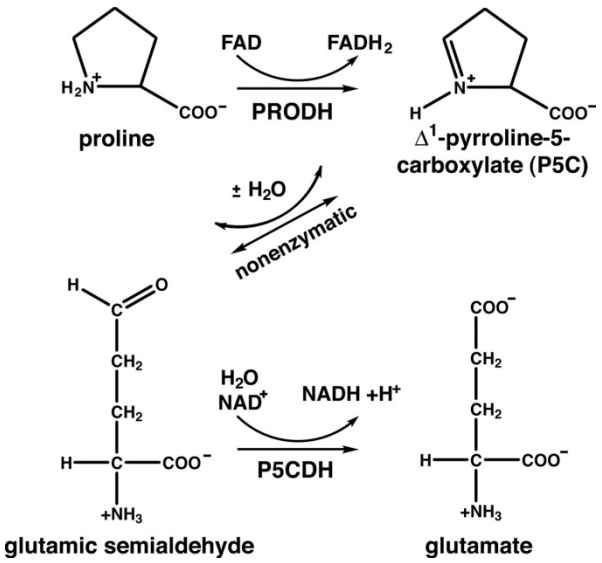
acyl-CoA dehydrogenase

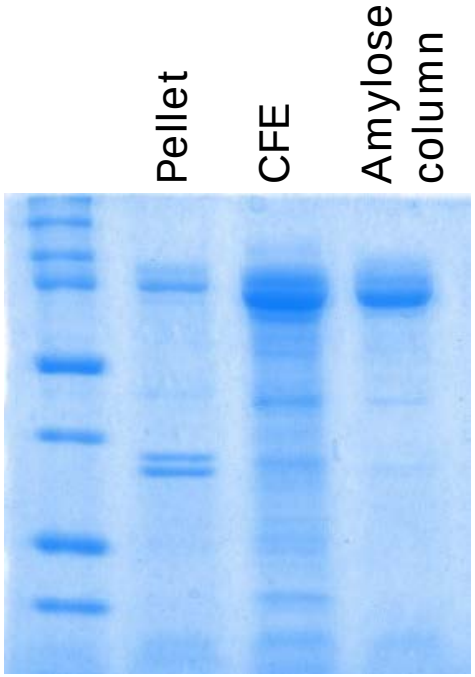
$FADH_2$-dependent halogenase

Prolyl dehydrogenase (PRODH)

Thermophilic proline dehydrogenase

- PRODH from *Thermus thermophilus*
- High overproduction of MBP-tagged flavoenzyme
- Active as fusion and after removal of MBP tag
- Enzyme fully stable at 80 °C

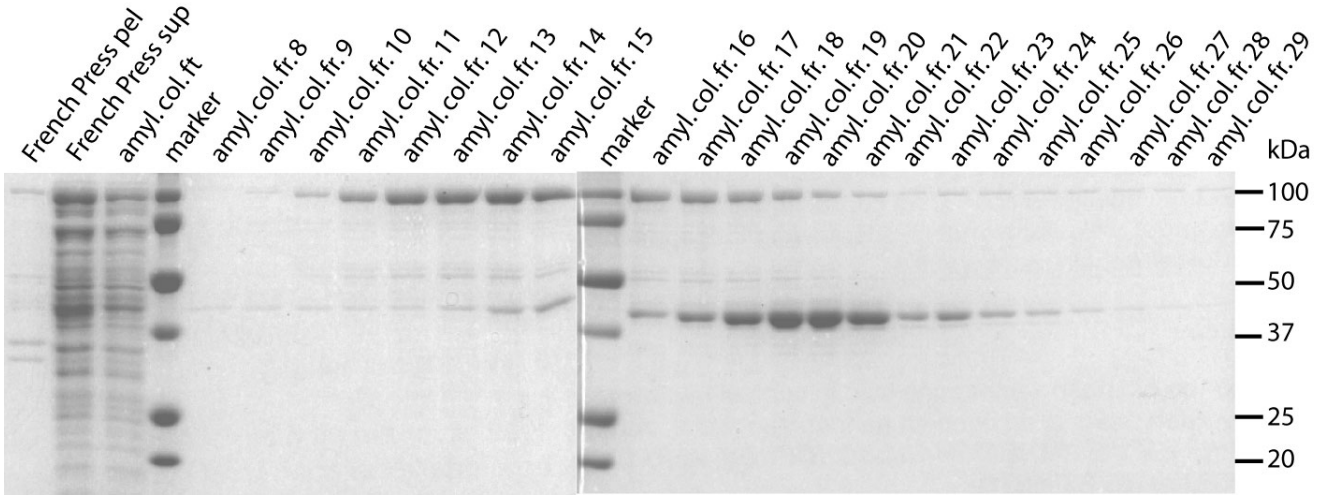




Prolyl dehydrogenase (PRODH)

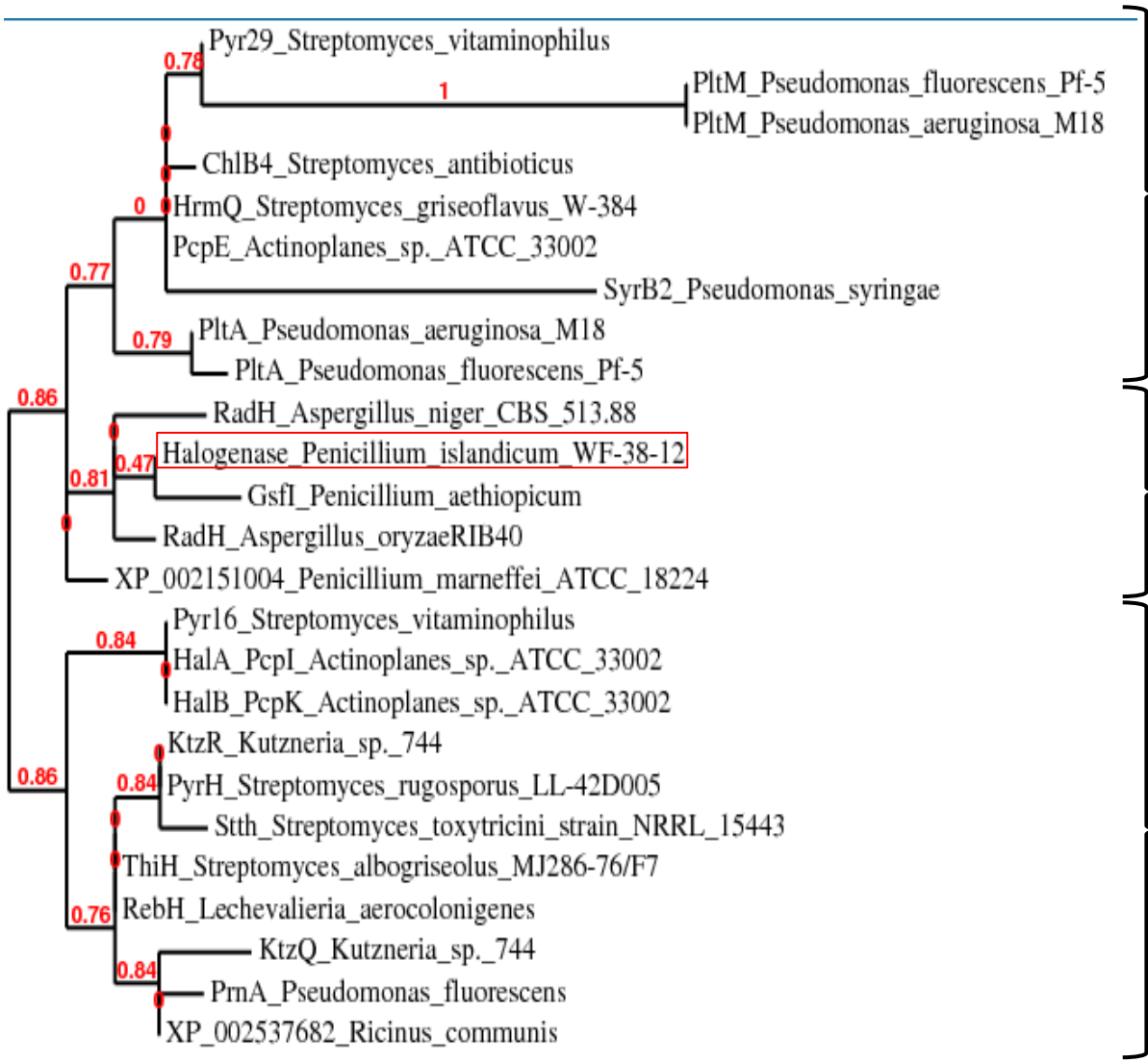
Fungal proline dehydrogenase

- 3 Putative PRODH genes in *Penicillium islandicum*
- One PRODH produced in soluble form
- One-step purification of MBP-tagged flavoenzyme
- Active as fusion and after removal of MBP tag



First fungal prolyl dehydrogenase

Flavin-dependent halogenases



pyrrole halogenases

putative halogenases from fungi

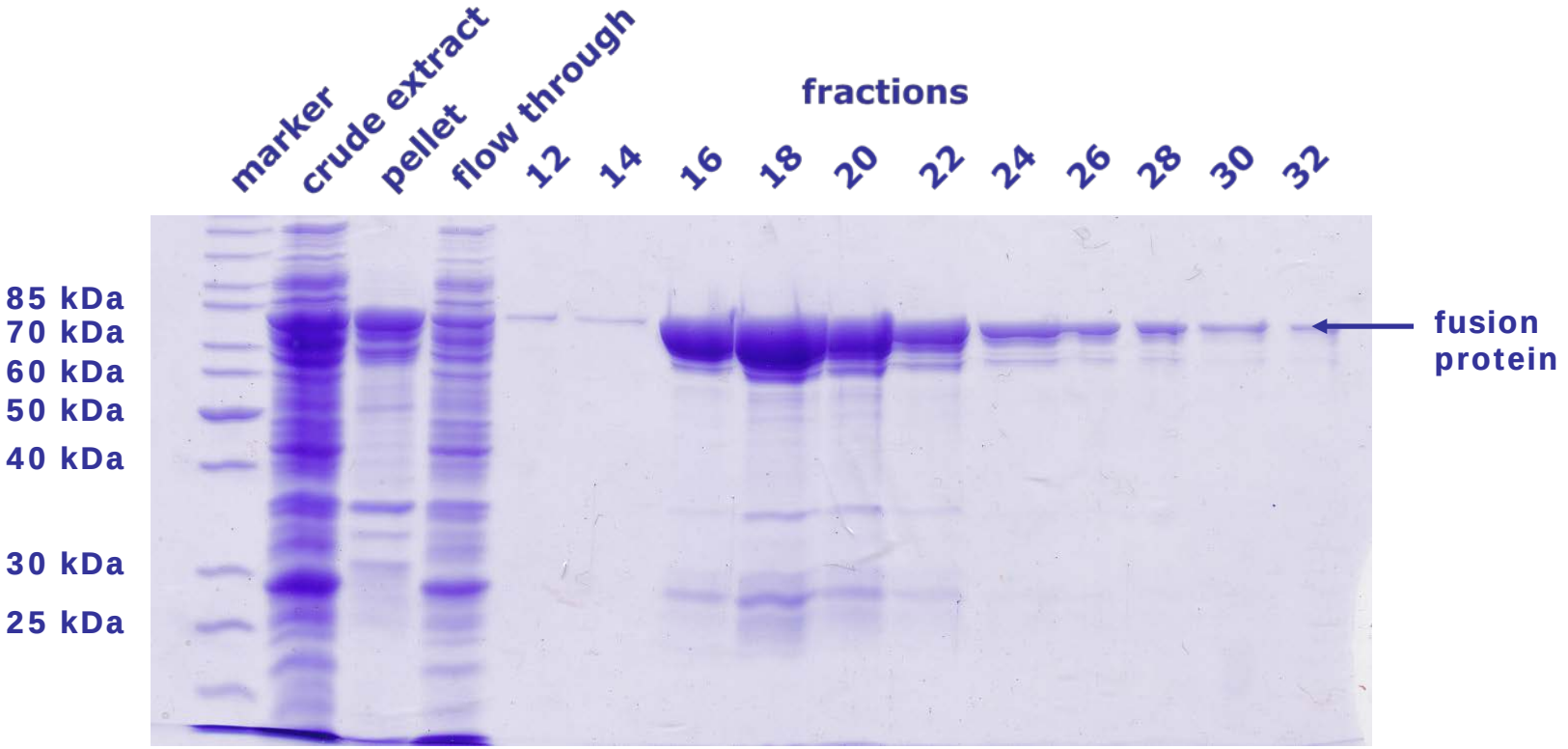
tryptophan halogenases

Halogenase from *P. islandicum*

Purification of the halogenase

50 µM IPTG, over night at 20 °C

GST fusion protein: 83.6 kDa



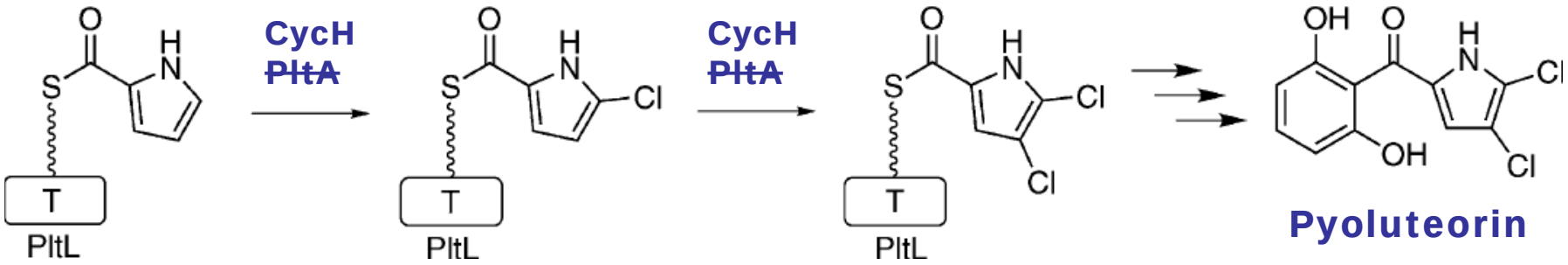
Halogenase can be purified using a maltose-binding protein tag.

Demonstration of halogenase activity

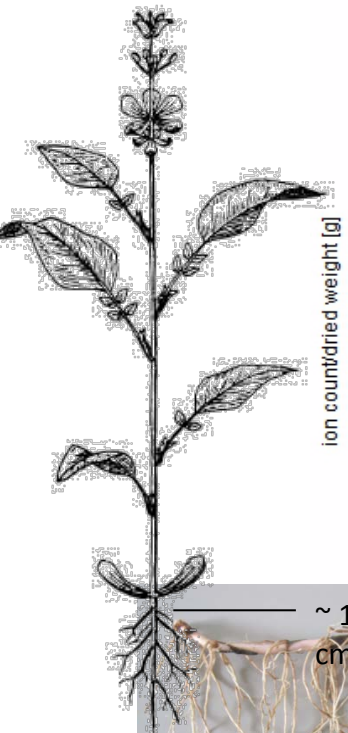
Activity assay using a chemically synthesised substrate mimic has not been successful, yet.

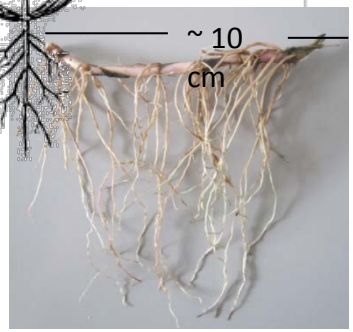
Complementation of pyoluteorin biosynthesis in *Pseudomonas fluorescens*.

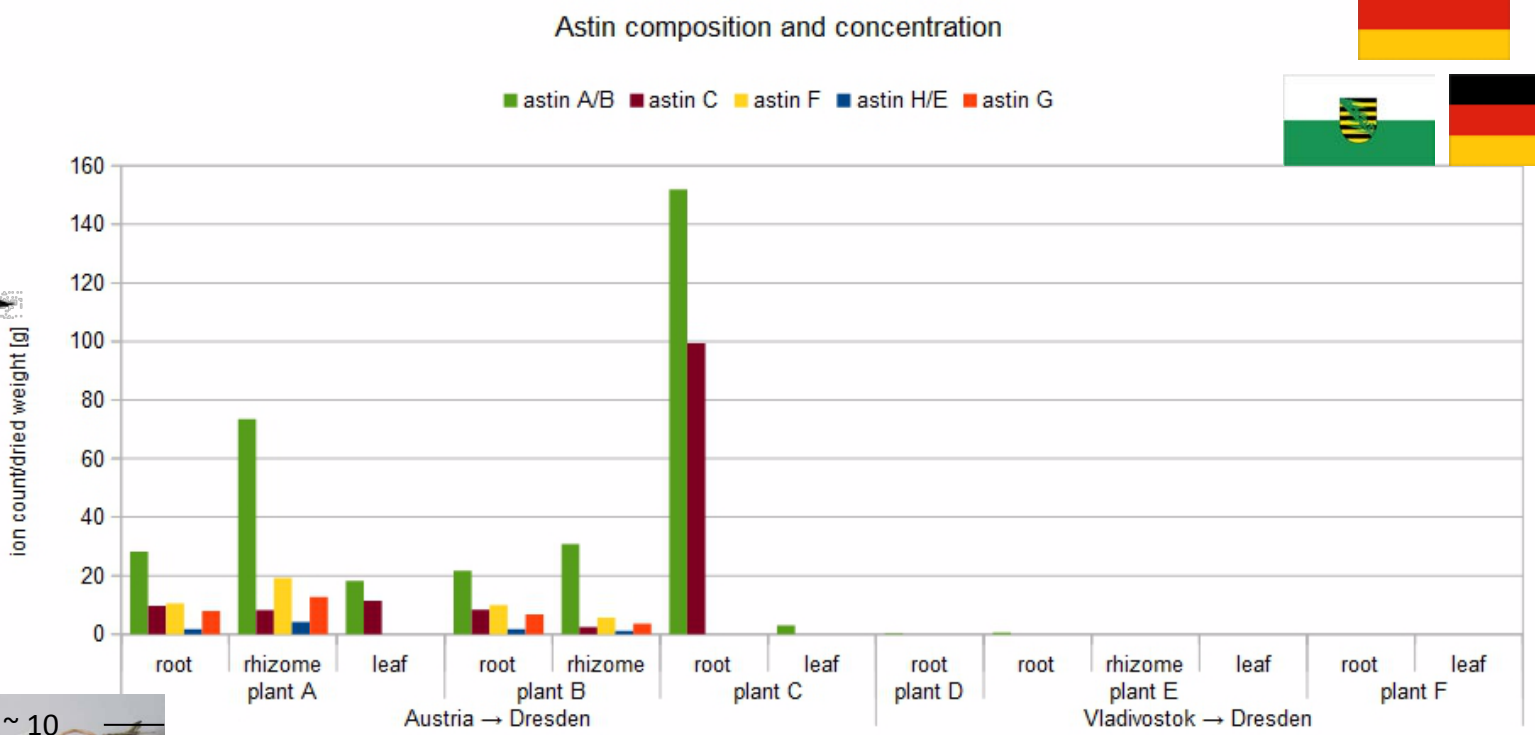
The pyrrole halogenase gene *plt*A is inactivated and will be substituted by the halogenase gene *cycH* from the cyclochlorotine producer *P. islandicum* to show halogenating activity *in vivo* by complementing pyoluteorin biosynthesis.



Localisation of astins in *A. tataricus*



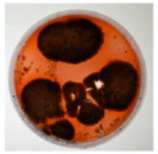




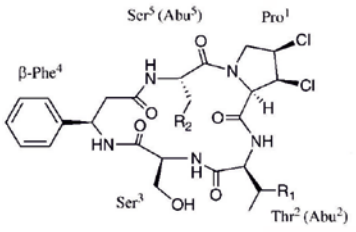
→ **Presence of astins depends on plant origin**

Astins, plant or fungal metabolites?

P.islandicum (10 strains)

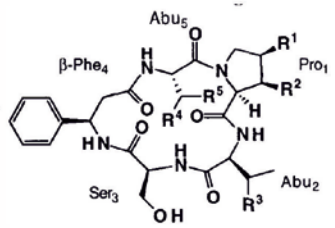


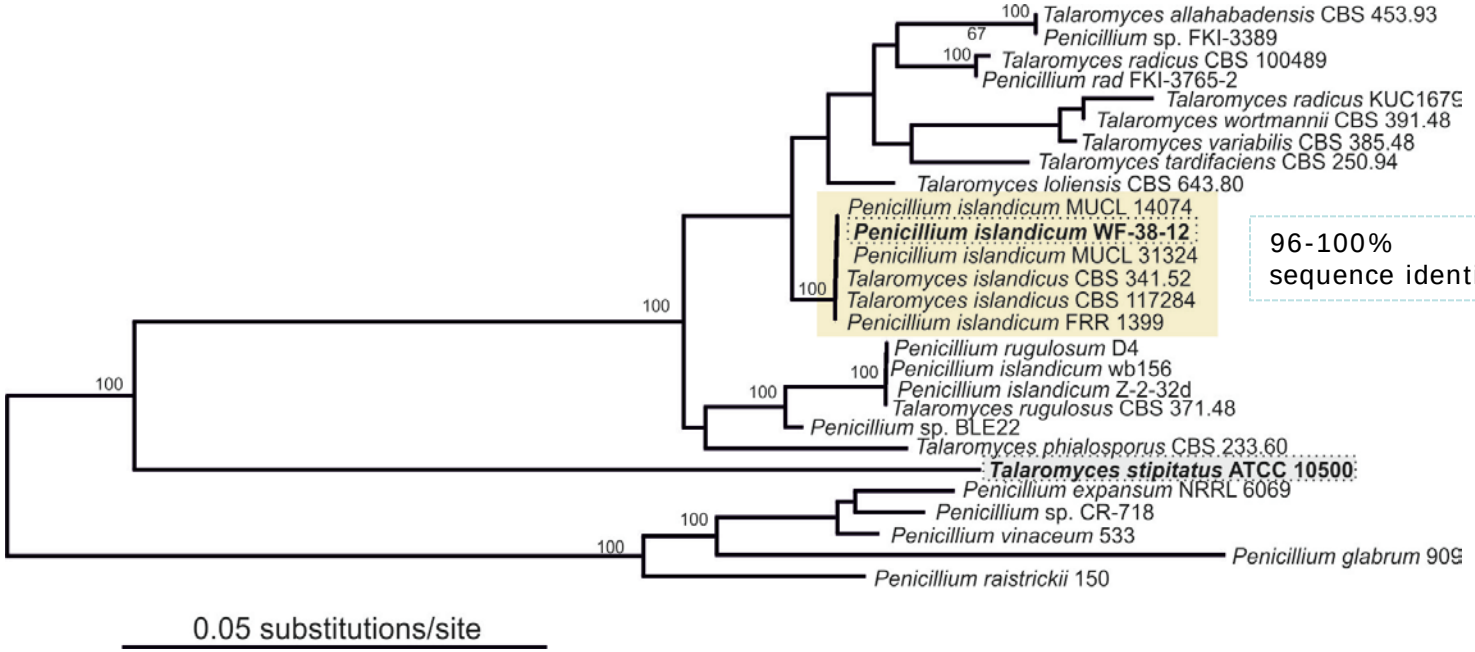
cyclochlorotines



A.tataricus (2 plants)

astins





96-100% sequence identity

Cyclochlorotines were identified from strains of *P. islandicum* isolated from soil or spoiled food; none of them produces astins.

Astins, plant or fungal metabolites?

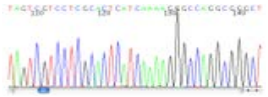
A. tataricus **(Austria) roots**

wash

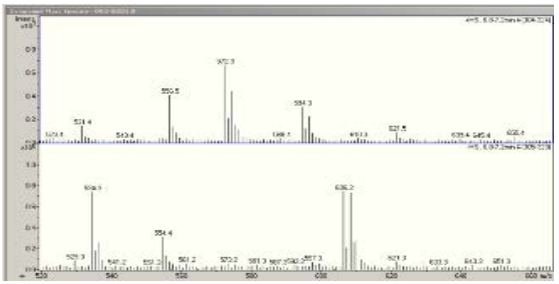
surface sterilize

fungal endophytes

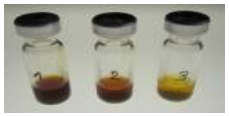
isolate

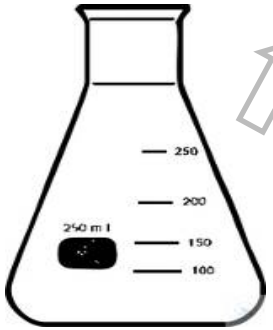


ribosomal DNA



HPLC-MS analysis of extracts





Astins could not be detected in any of the fungi isolated from *A. tataricus* roots.

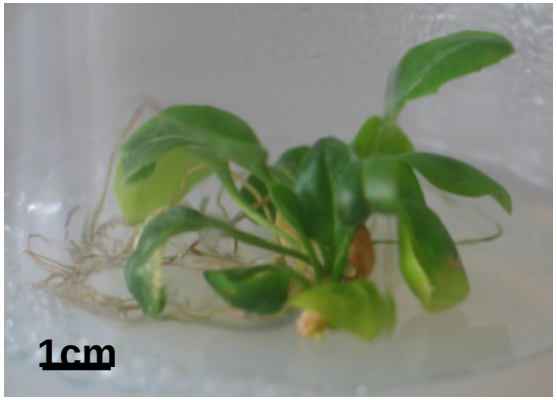
Generation of sterile plants

Is there astin production in sterile plants?

Cultivation of sterile seeds on MS medium without any hormones

Cultivation of sterilised inflorescence tissue on MS medium (naphthyl acetic acid or benzyl amino purine) to induce regeneration of small, sterile plants

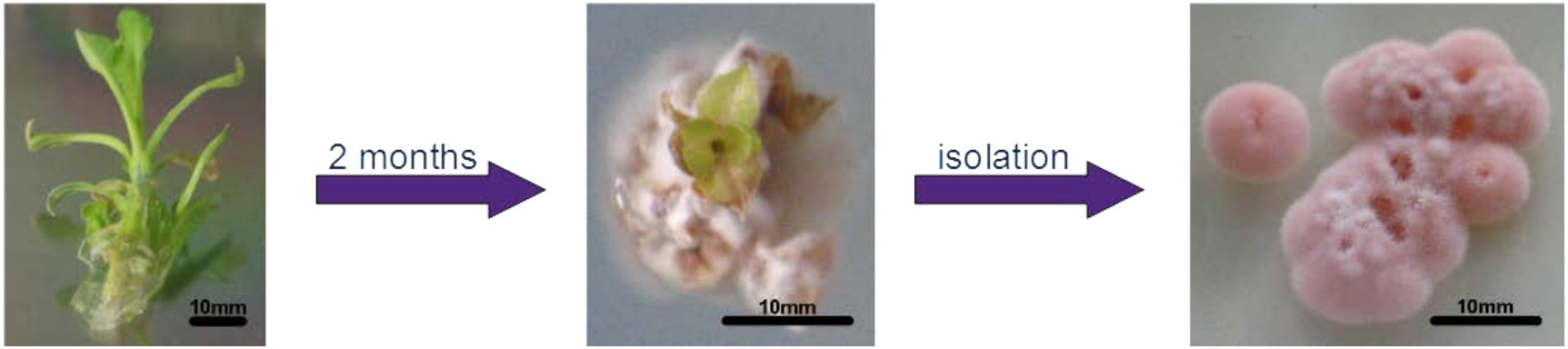
These plants turned out to be not really sterile!



Sterile plant from sterilised seeds

Germinating plant from inflorescence axis on MS medium + 1µg/ml BAP

Growth of “sterile“ plants

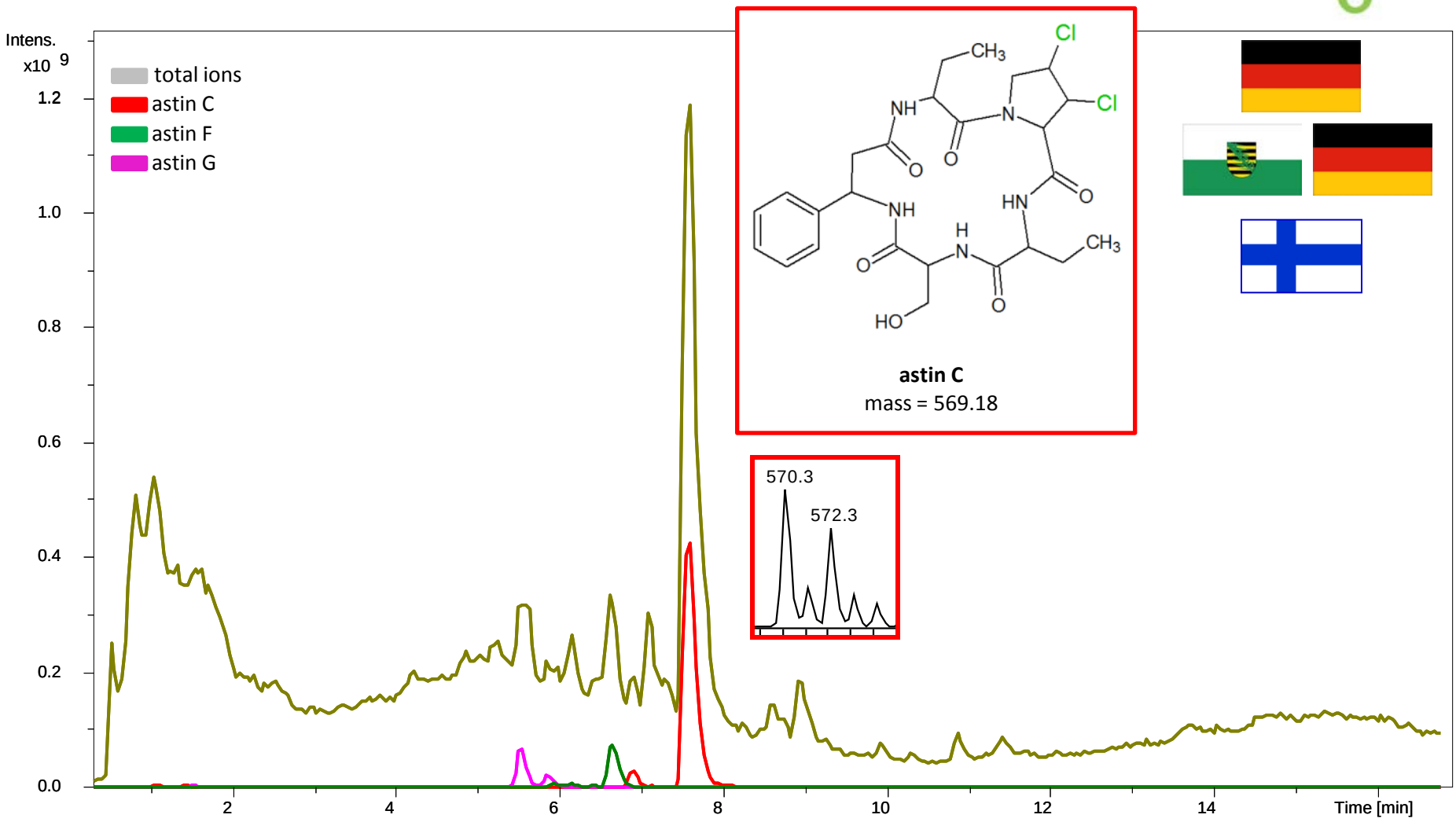


Generation of sterile *in vitro* cultures of *Aster tataricus* (Austrian) via hormone treatment (auxin and cytokinin for inducing shoot and roots)

After a few months, growth of a pink fungus could be observed. This fungus could not be removed by treatment with antimycotica like amphotericin.

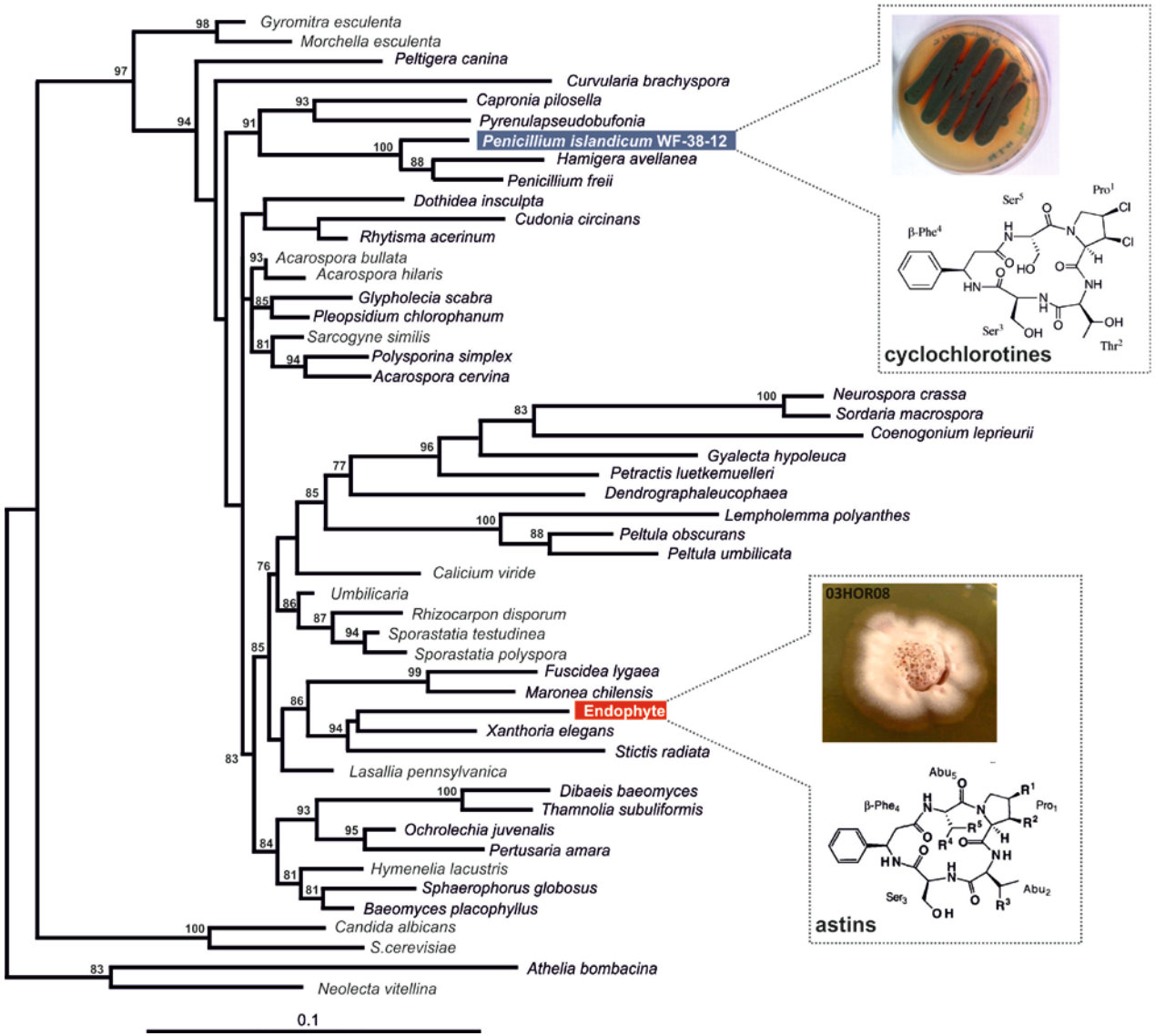
Isolation of a novel fungus

Production of astins by the novel fungus



Astin C is the main compound. Astins produced by the new fungus are identical to those isolated from dried *A. tataricus* roots.

An endophyte which produces astins



Astins were identified in a fungal endophyte isolated from *A. tataricus* which is not closely related to *P. islandicum*.

Genome sequencing of endophyte

Astin producing endophyte	vs.	*Penicillium islandicum*

genome parameters

length:	28 MB	length:	34 MB
GC content:	53 %	GC content:	45,22 %
coverage:	57 fold	coverage:	41 fold
scaffolds:	395	scaffolds:	330

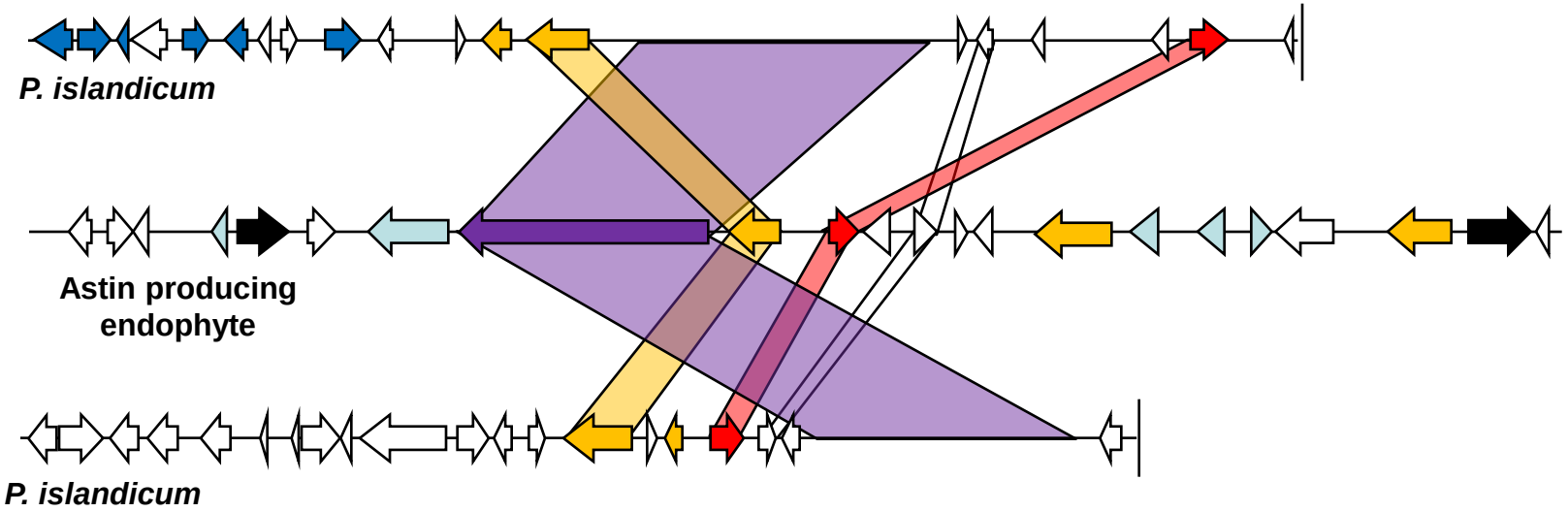
predicted secondary metabolite gene clusters (by antiSMASH)

PKS:	13	PKS:	19
NRPS:	5	NRPS:	17
	(1/5 is 5-modular)		(2/17 are 5-modular)
NRPS-PKS:	1	NRPS-PKS:	8
terpene:	5	terpene:	4
other:	9	lantipeptide	1
sum	**33**	other:	11
		sum	**60**

Partial astin gene cluster

Comparison of gene clusters

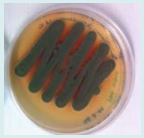
Genes with high similarity



There is a gene cluster present in the new fungus with high similarity to two gene clusters in the cyclochlorotine producer *P. islandicum*

Summary

Modified proposal



Cyclochlorotines
P. islandicum

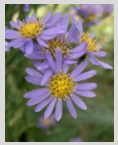
Genome sequencing

Identification of NRPS gene cluster

Cloning of individual genes

Enzymatic activity and gene inactivation

Proposal

Astins
A. tataricus

Identification of individual biosynthetic genes

Expression and enzymatic activity

Construction of gene cluster

Heterologous expression

Production of astins

Bioactivities

What we achieved

Astins

Isolation of a novel fungus producing astins

Draft genome sequence

Astins can now be produced by fermentation