Pine Wilt Disease in Europe

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Searching for resistance genes to the PWN using SSH and High Throughput Screening

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CBQF Centro de Biotecnologia e Química Fina

Plantech

The PWN

Ethiology

- *Bursaphelenchus xylophilus*
- Vector: *Monochamus spp*.
- Main symptom: wilting of leaves
- Primary host in Portugal: *Pinus pinaster*
- Less susceptible species?
  - *Pinus pinea*

Gene regulation

- Gene induction: hours
- Visible symptoms: days-weeks

What are the molecular players involved in disease response and plant defense?

Previous works

- Shin et al (2009) Identification of genes upregulated by pinewood nematode inoculation in Japanese red pine. Tree Physiology 29: 411-421
  - SSH -21hai
  - upregulated genes from PWN-inoculated Japanese red pine (Pinus densiflora)
  - 33 DEGs and 2778 ESTs
  - pathogenesis-related proteins, pinosylvin synthases and metallothioneins

Previous works

- M. Nose & S. Shiraishi (2010) Comparison of the gene expression profiles of resistant and non-resistant Japanese black pine inoculated with pine wood nematode using a modified LongSAGE technique. Forest Pathology 41: 143-155
  - 20 818 tags
  - 14 up-regulated (PR 2 and 4, osmotin, lipoxygenase, chalcone synthase) and 9 down-regulated (eukaryotic translation initiation factor, translationally controlled tumor protein, xyloglucan endotransglycosylase)
  - 38 in resistant pine (catalase, dienelactone hydrolase)
  - 25 in non-resistant pine (PR 1, 2, 3, and leucoanthocyanidin dioxygenase).

Previous works

- N. HAMAMOUCH1, C. LI, P. J. SEO, C. PARK, E.L. DAVIS (2010) **Expression of Arabidopsis pathogenesis-related genes during nematode infection**. Molecular Plant Pathology 12: 355-364
  - The expression pattern of pathogenesis-related genes *PR-1* to *PR-5* was examined in the roots and leaves of *Arabidopsis thaliana* plants on infection with beet-cyst (*Heterodera schachtii*) and root-knot (*Meloidogyne incognita*) nematodes
  - Over-expression of *PR-1* reduced infection by both *H. schachtii* and *M. incognita*,
  - Over-expression of *PR-3* reduced host susceptibility to *M. incognita* but had no effect on *H. schachtii* parasitism

Previous works

- Kuroda et al. (July 2011) The expressed genes of Japanese red pine (*Pinus densiflora*) involved in the PWD severity. IUFRO Tree Biotechnology, Brazil
  - Megasort Megabead technology
  - 16 upregulated (transporters, thaumatin like proteins, PR proteins)
  - 13 downregulated

Our goal

1) To utilize **SSH** and **454 Pyrosequencing** to identify genes differentially expressed in *P.pinaster* and *P.pinea*
   - SSH: 3 hai
   - 454: 24 hai

2) To select a candidate resistance gene for over-expression in *P. pinaster*

SSH technique

Analysis of differentially expressed genes:

> Forward subtraction: *P. pinaster*+HF

> Reverse subtraction: *P. pinea*+HF

Differentially expressed genes

> Unsubtracted Tester: *P. pinaster*+HF

> Unsubtracted Driver: *P. pinea*+HF

SSH technique

Forward Subtraction

↓

12 sequences

↓

No homology in BlastN and BlastX searches of NCBI

Reverse Subtraction

↓

33 sequences

↓

5 had hits in BlastN and BlastX searches of NCBI

↓

Similar to putative **histones H4** of *Picea* spp.

- Constituent of chromatin
- Present in the nucleosome
- Undergoes diverse post-translational modifications

SSH technique

Forward Subtraction	Reverse Subtraction
↓	↓
12 sequences	33 sequences
↓	↓
No homology in BlastN and BlastX searches of NCBI	5 had hits in BlastN and BlastX searches of NCBI
↓	↓

Is H4 histone protein expression one of the molecular players involved in the lower susceptibility of *P. pinea* when compared to the more susceptible species *P. pinaster*?

SSH technique

Unsubtracted Tester (*P. pinaster*)
Putative alfa tubulin
Possible cytosolic Fe-S protein
Likely cytochrome oxidase subunit I
Putative thioredoxin
Putative translation elongation factor-1
Likely FMN-dependent alpha-hydroxyacid
Possible phenylalanine ammonia lyase
Non-specific lipid-transfer protein type 1
Hypothetical xyloglucan endotransglycosylase
Genes of (or related to) RNA recognition motif

SSH technique

Unsubtracted Driver (*P. pinea*)

Putative clavata-like receptor

Putative protein belonging to Class-II DAHP synthetase family

Possible s-adenosyl methionine synthetase 2

Likely copper resistance protein

mRNA up-regulated during drought stress

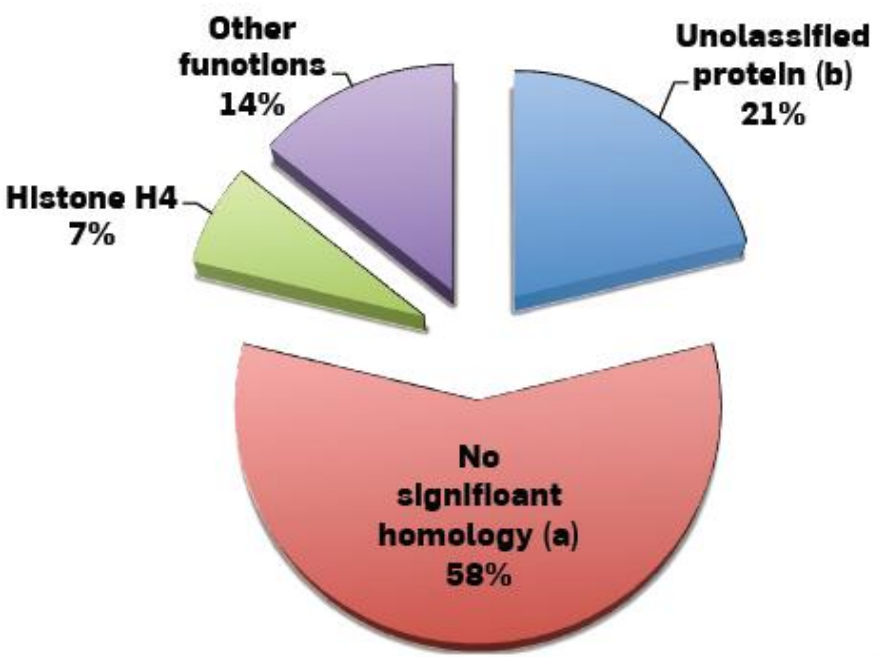
Probable RNA recognition motif

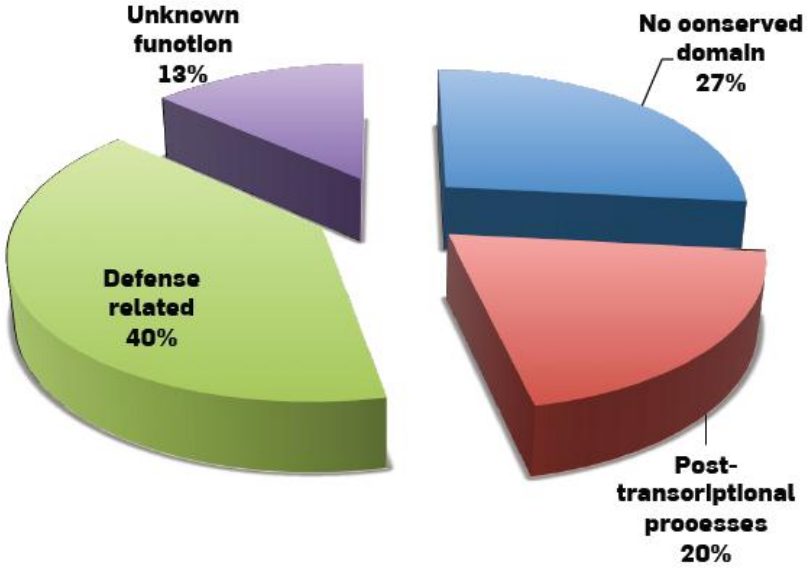
Sm-like protein

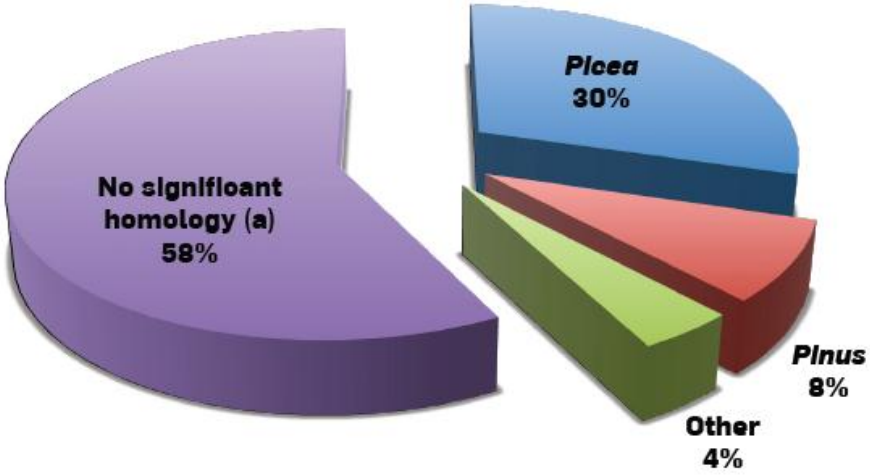
Protein similar to one belonging to DUF231 *Arabidopsis* proteins

NifU-like protein

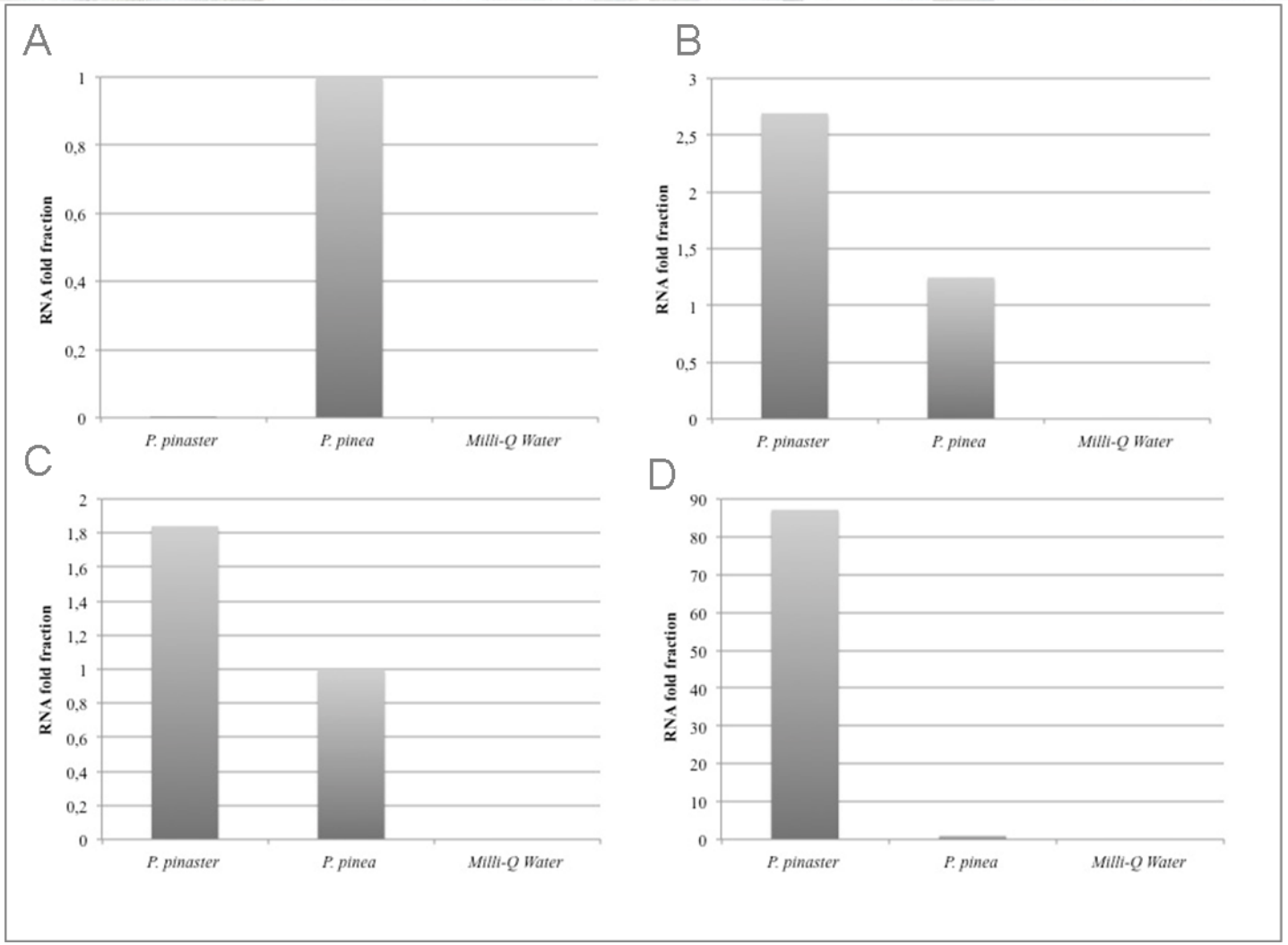
SSH technique







Gene expression confirmation



A) *P. pinea* Histone B) *P. pinaster* Unknown C) *P. pinaster* drought stress D) PAL

SSH: conclusions

- The fact that 58% of the isolated sequences didn't have a significant homology in the NCBI database reveals that publicly available databases have very little information on the *Pinus* spp.genome sequences;
- 40% of the expressed genes were related to defense mechanisms;
- Oxidative stress was found to be a very important defense mechanism triggered by the infection;
- Histone H4 was the differentially expressed gene by *P. pinea*,which might contribute to its apparent resistance to the disease;

454 Pyrosequencing

- Main objective: pyrosequencing of transcriptome of *P. pinea* and *P. pinaster* infected and non-infected with *B. xylophilus*
- Strategy: inoculation of 2 year old plants and collecting samples at 24h
- Handling:
  - RNA extraction
  - Synthesis of ds cDNA from RNA
  - Pyrosequencing of cDNA libraries

454 Pyrosequencing

	Region 1 *P. pinaster*+HF	Region 2 *P. pinaster* control
Number reads	650,733	574,456
Total number of bases	268,683,993	235,513,329
Average length	412.9	410

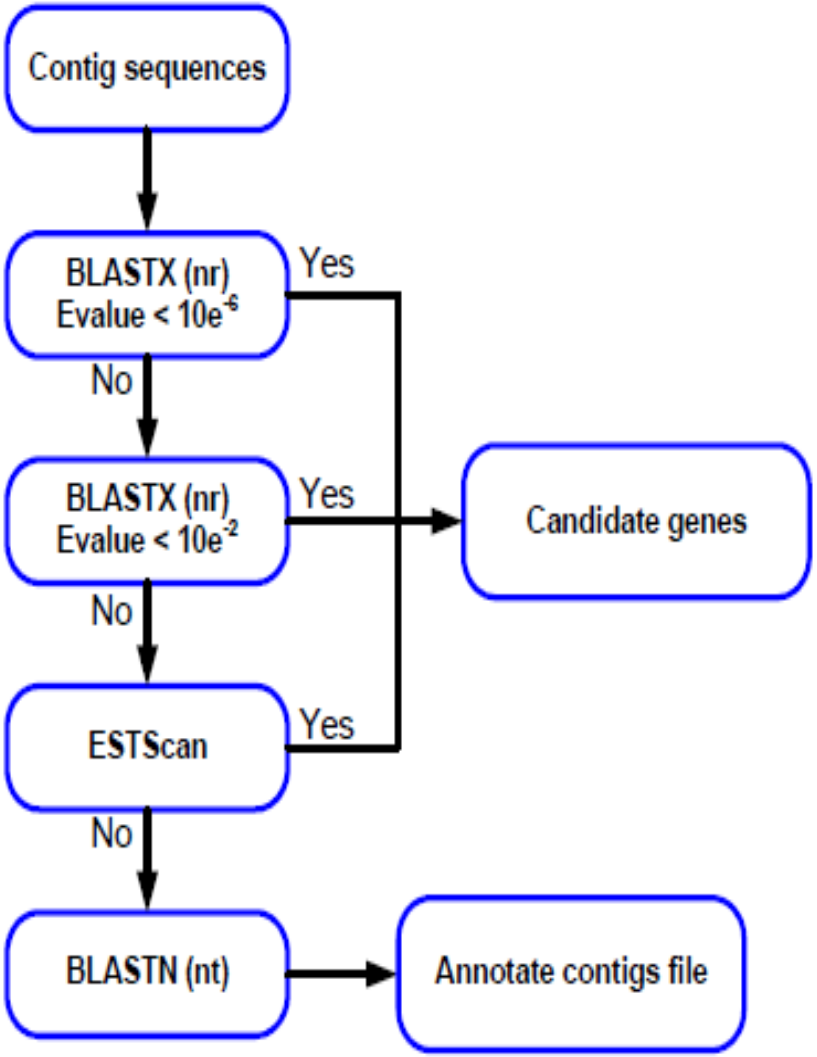
	Region 1 *P. pinea*+HF	Region 2 *P. pinea* control
Number reads	494,604	366,237
Total number of bases	210,489,814	149,298,262
Average length	425.6	407.7

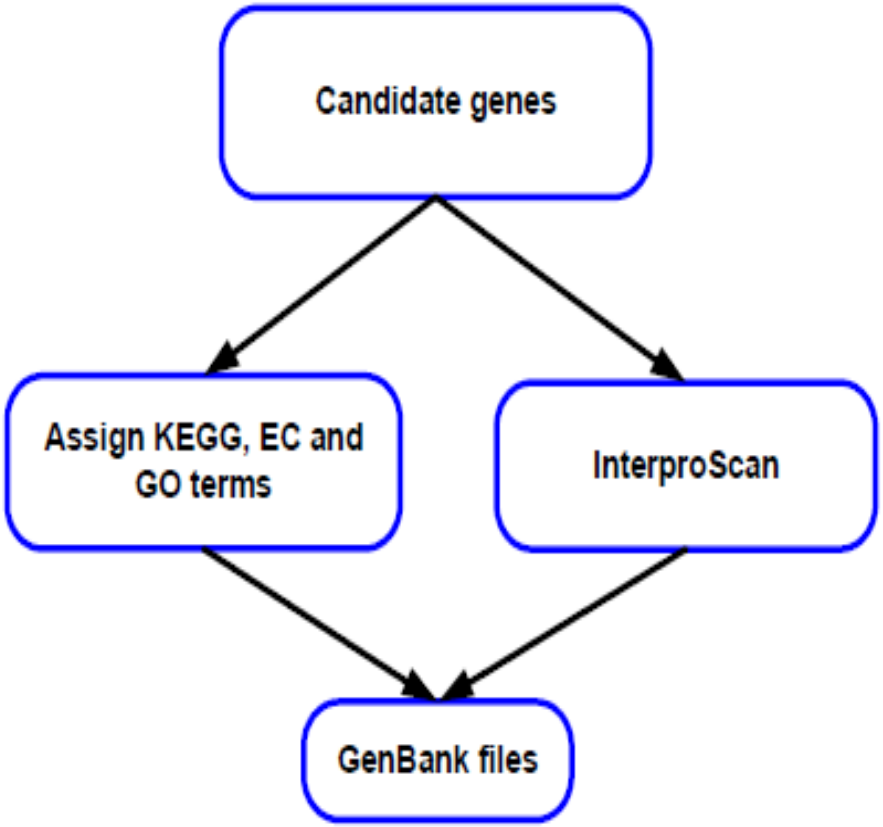
Assembly summary

(Ex: *Pinus pinaster* + nematode)

	Number
Number of Reads	479,467
Total Bases	153,567,000
Average read length after trimming	320.29
Number of contigs	34,739
Average contig length	587.85
Range of contig length	40-4,856
Number of singletons	1,814
Number of Contigs with 2 reads	8,427
Number of Contigs with >2 reads	24,498

Annotation scheme

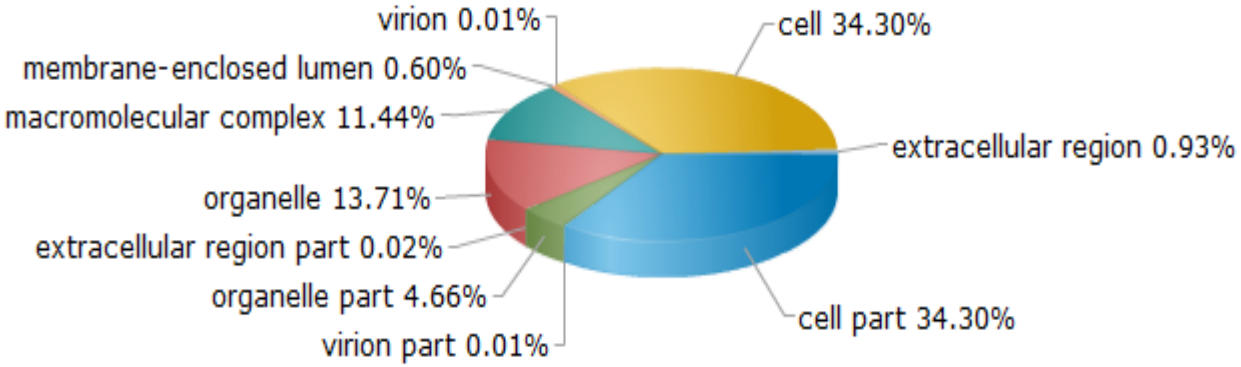




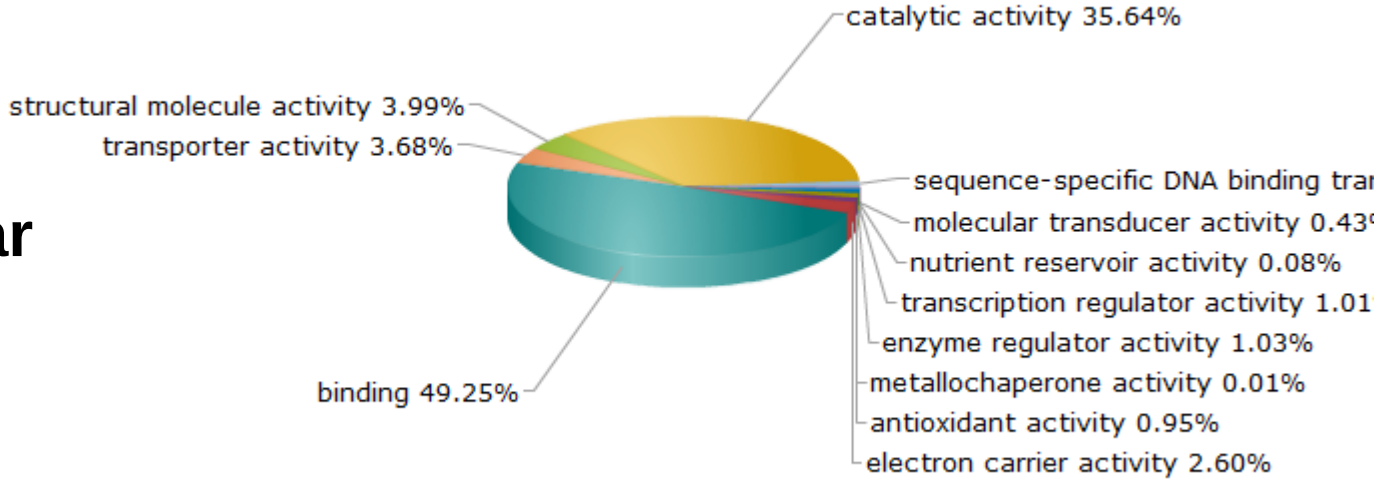
GO Annotation

(Ex: *Pinus pinaster* + nematode)

GO cellular component



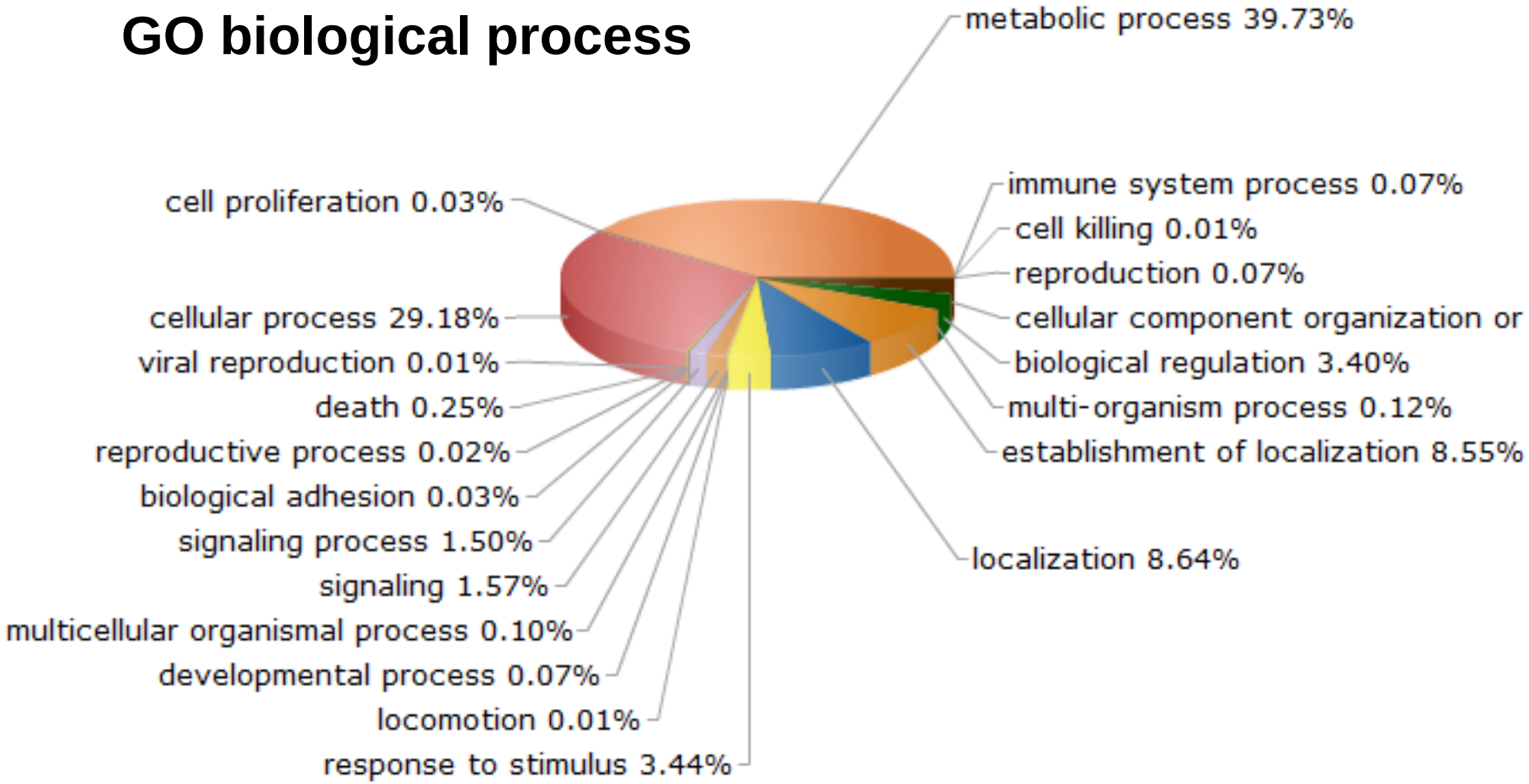
GO molecular function



GO Annotation

(Ex: *Pinus pinaster* + nematode)

GO biological process



Website

Logout | Login: (Marta Vasconcelos)

Available Projects

Transcriptomics

biocant

Available projects

P.pinae(sample MV_04)
P.pinae (sample MV_03)
P.pinaster (sample MV_02)
P.pinaster (sample MV_01)
Pinus samples (MV_01, MV_02)
Pinus samples (MV_01, MV_03)
Pinus samples (MV_02, MV_04)
Pinus samples (MV_03, MV_04)

Select

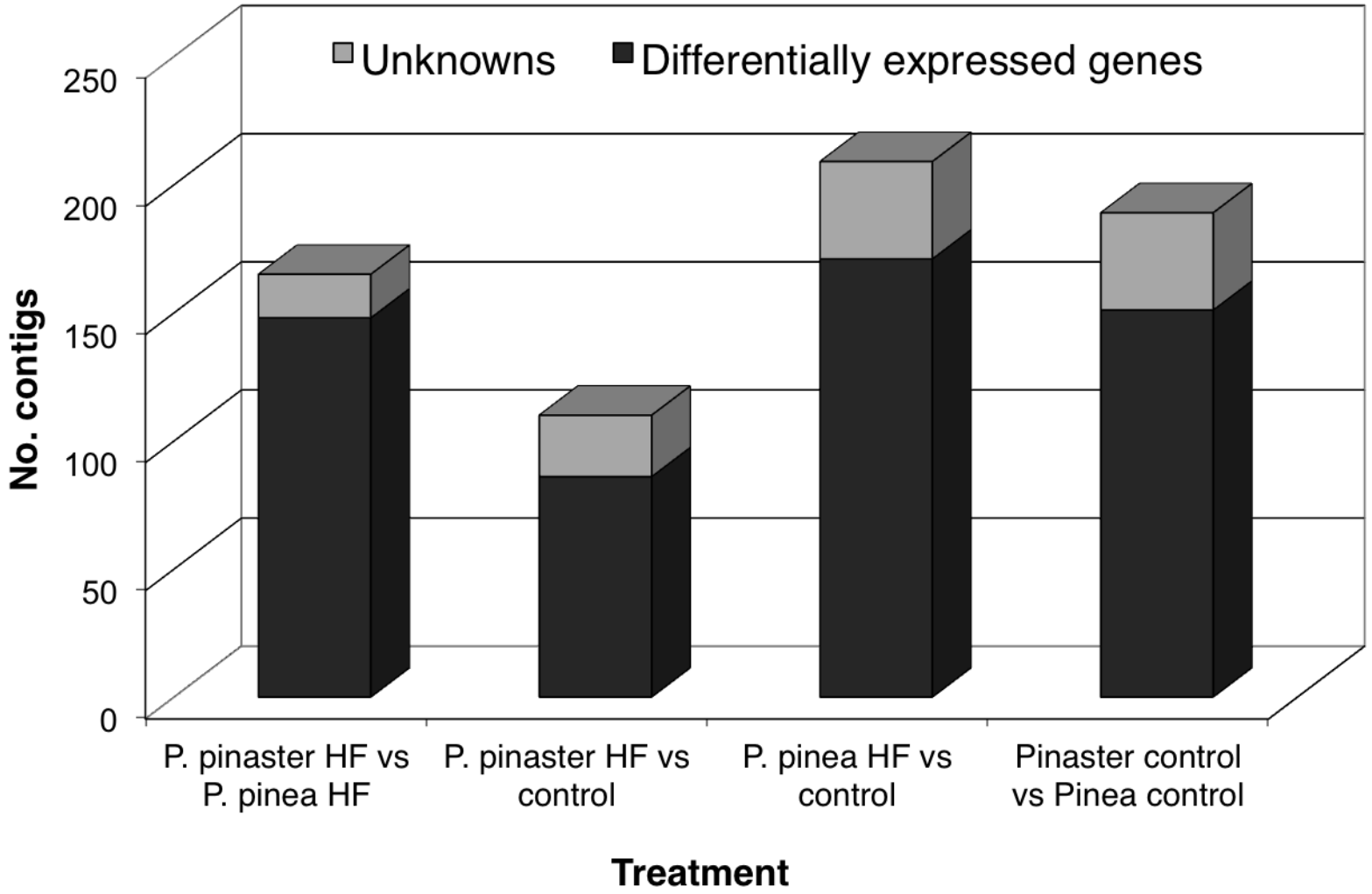
Press Ctrl+mouse to choose more than one project simultaneously

pine_nematode

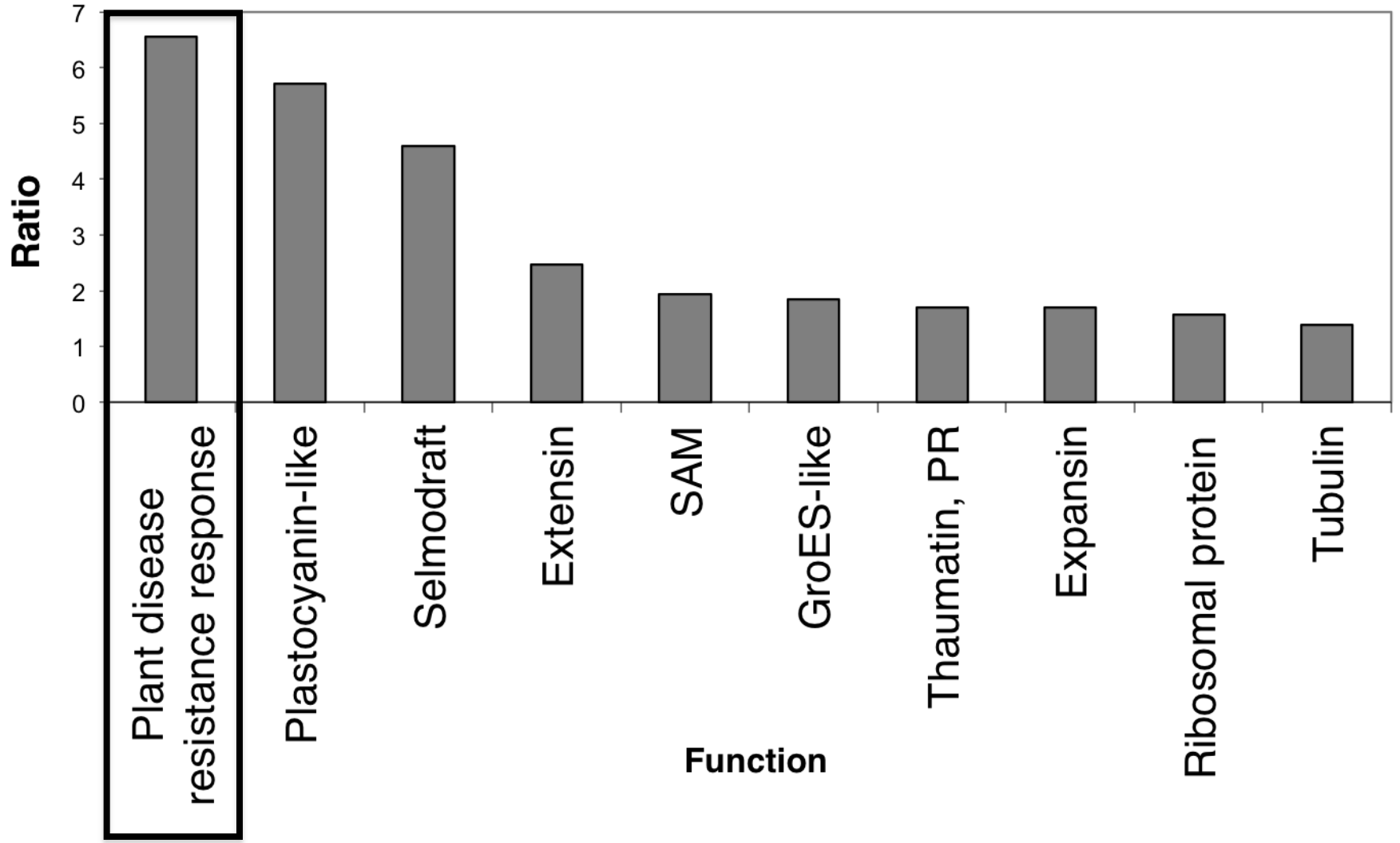
Sample Collected:	Transcriptome of Pinus pinaster MV_01/MV_02
Region Collected:	Portugal
Specie:	Pinus pinaster
Description:	(Pinus pinaster + HF)/(Pinus pinaster + H2O). Assembled by gsAssembler 2.30
Is multiple sample:	True

Differentially expressed genes

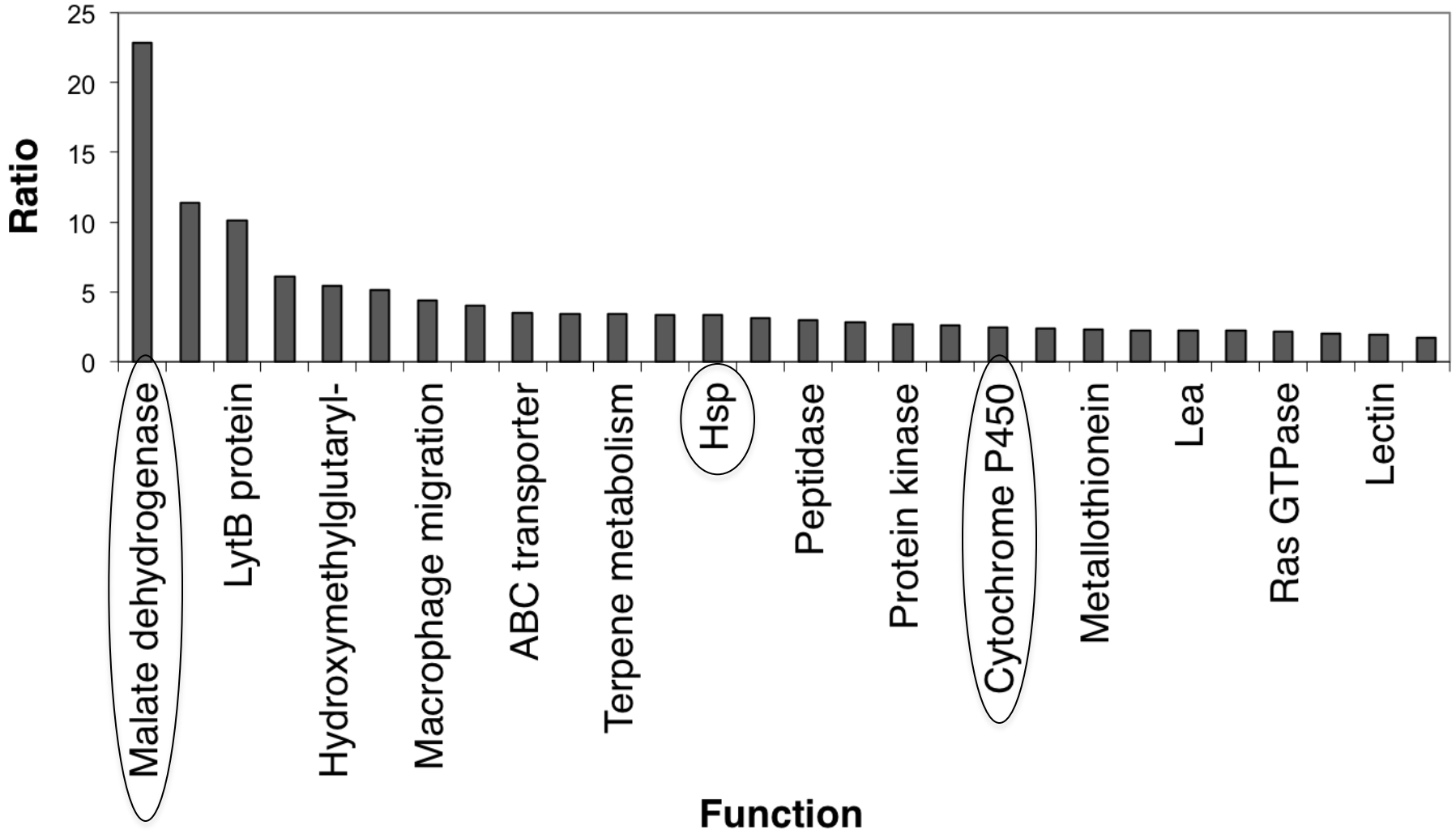
Four main comparisons:

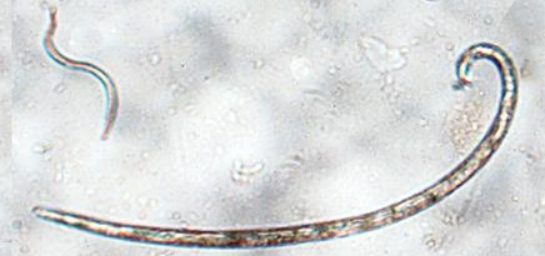


Up-regulated genes in *P. pinaster* HF vs control

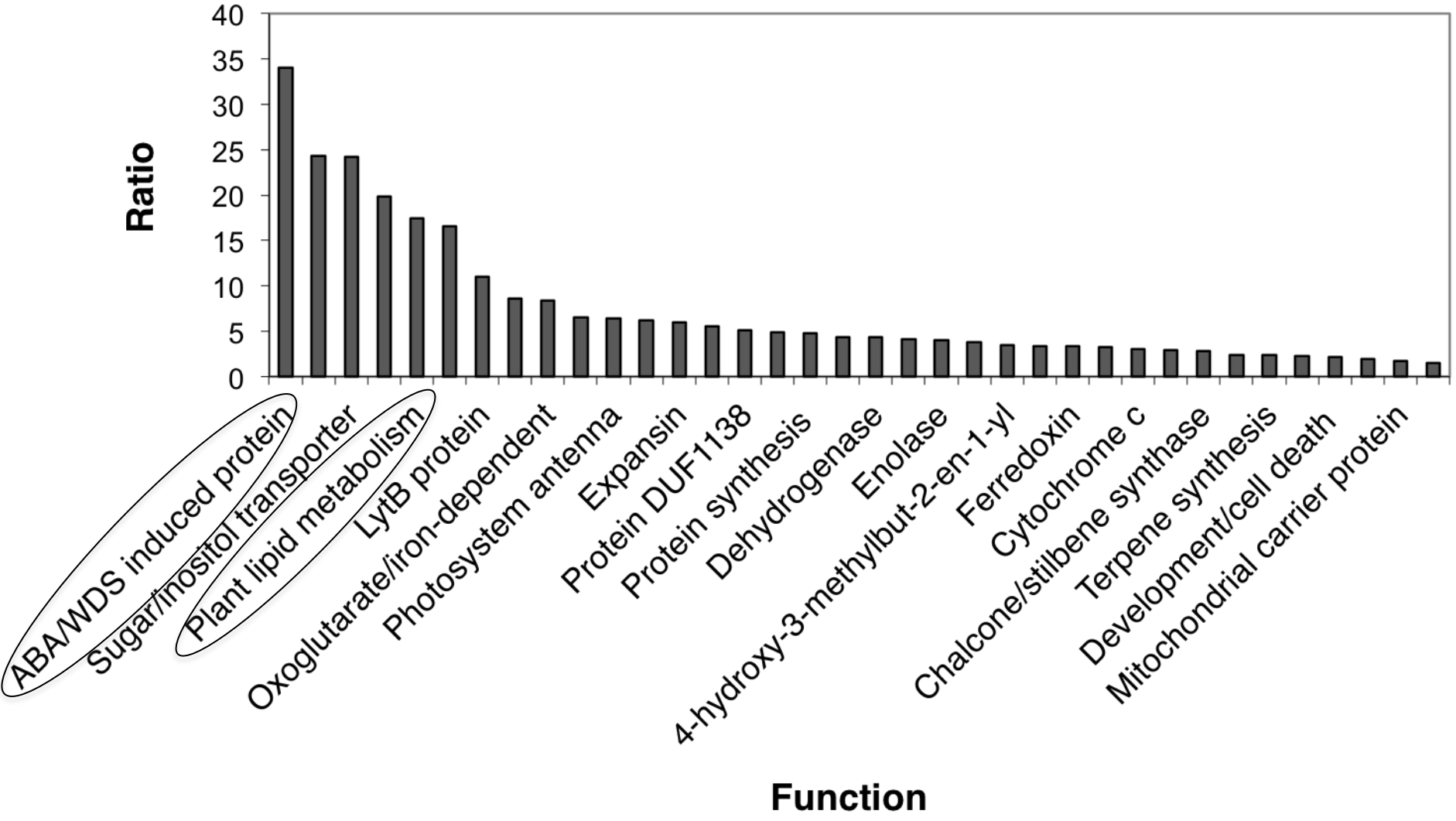


Up-regulated genes in control vs *P. pinaster* HF

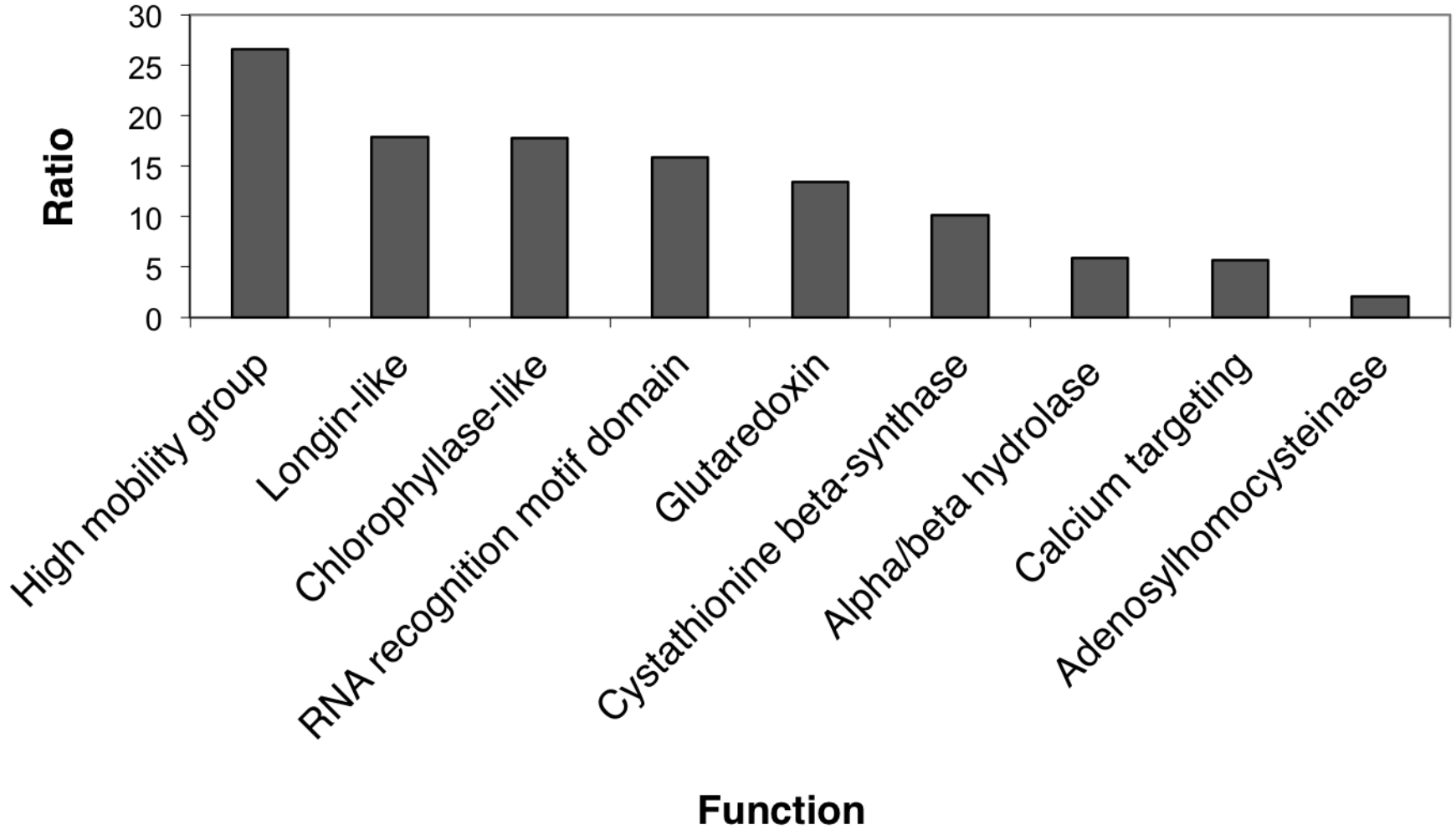




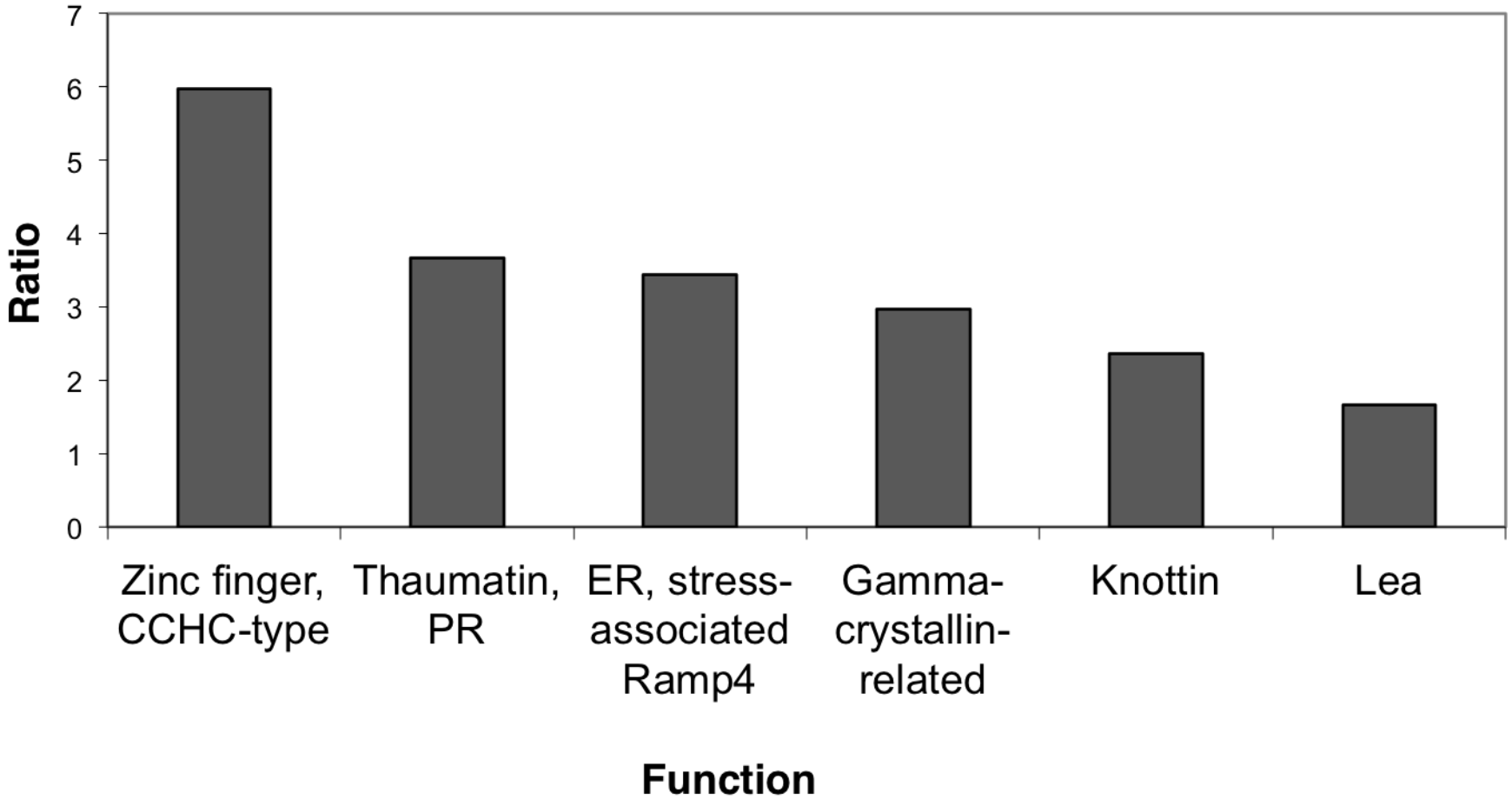
Up-regulated genes in *P. pinaster* HF vs P. pinea HF



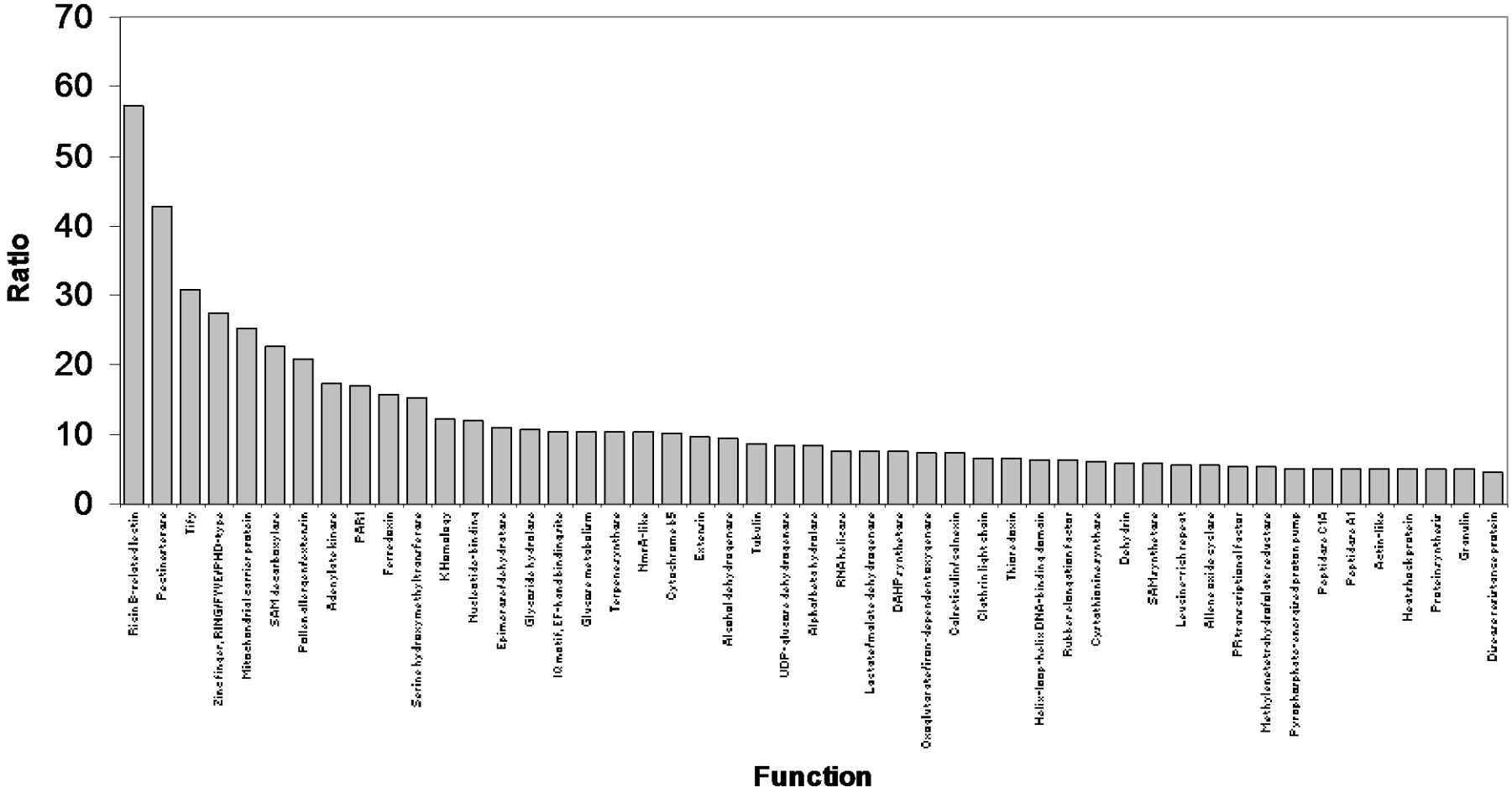
Up-regulated genes in *P. pinea* HF vs P. pinaster HF



Up-regulated genes in control vs *P. pinea* HF

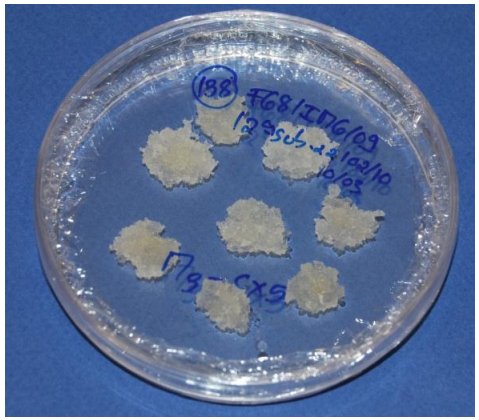


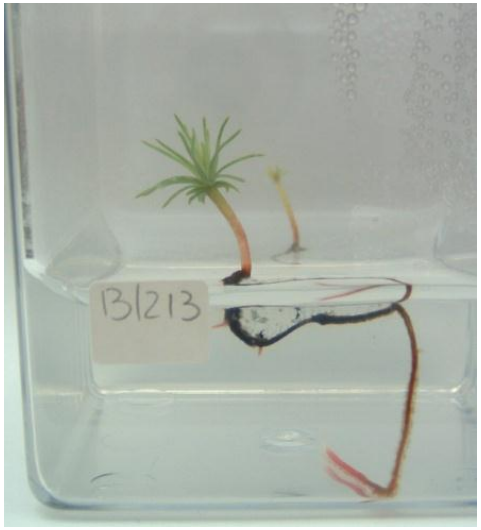
Up-regulated genes in *Pinea* HF vs control

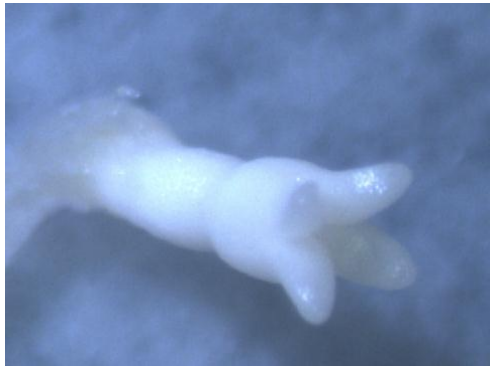


Conclusions Pyrosequencing

Preparing material for genetic transformation



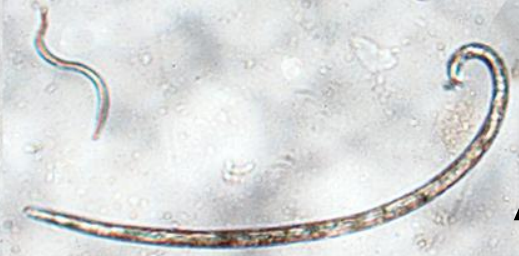




Photos kindly provided by Dra. Célia Miguel, IBET

Future work

- Validation of candidate genes via qRT-PCR
- Cloning of candidate gene
- Genetic transformation of Maritime pine in collaboration with IBET
- Testing transformed material for increased resistance to PWN



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- Biocant

Thank you for your attention