

II Reunião Científica de Sanidad Forestal

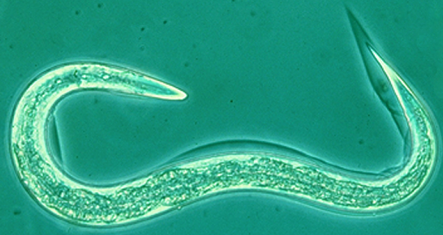
21 & 22 Setembro, 2011

UTILIZAÇÃO DA TÉCNICA DE SSH PARA DETECTAR A EXPRESSÃO DIFERENCIAL DE GENES DE DEFESA AO NEMÁTODE DA MADEIRA DO PINHEIRO

Carla Sancho dos Santos e Marta W. Vasconcelos

For more information about our research on PWN, please see Posters No. 1 & 2





PINE WOOD NEMATODE

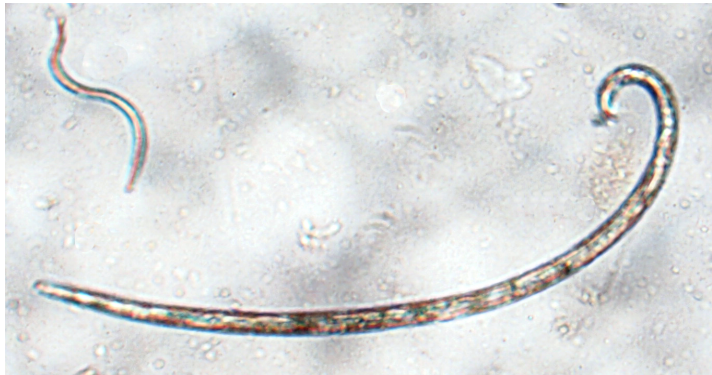


Figure 1 – Pinewood nematode
Bursaphelenchus xylophilus.

> Primary host:

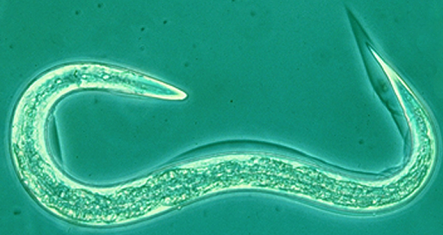
P. pinaster Ait. (also called maritime pine, or “pinheiro bravo”)

> Alternative hosts:

Pinus sylvestris and *Pinus halepensis*, but their distribution and abundance is limited in Portugal

> Less susceptible species?

P. pinea (also called stone pine, or “pinheiro manso”)



PWN IN PORTUGAL



Resultados das amostras
a 15 Julho 2008

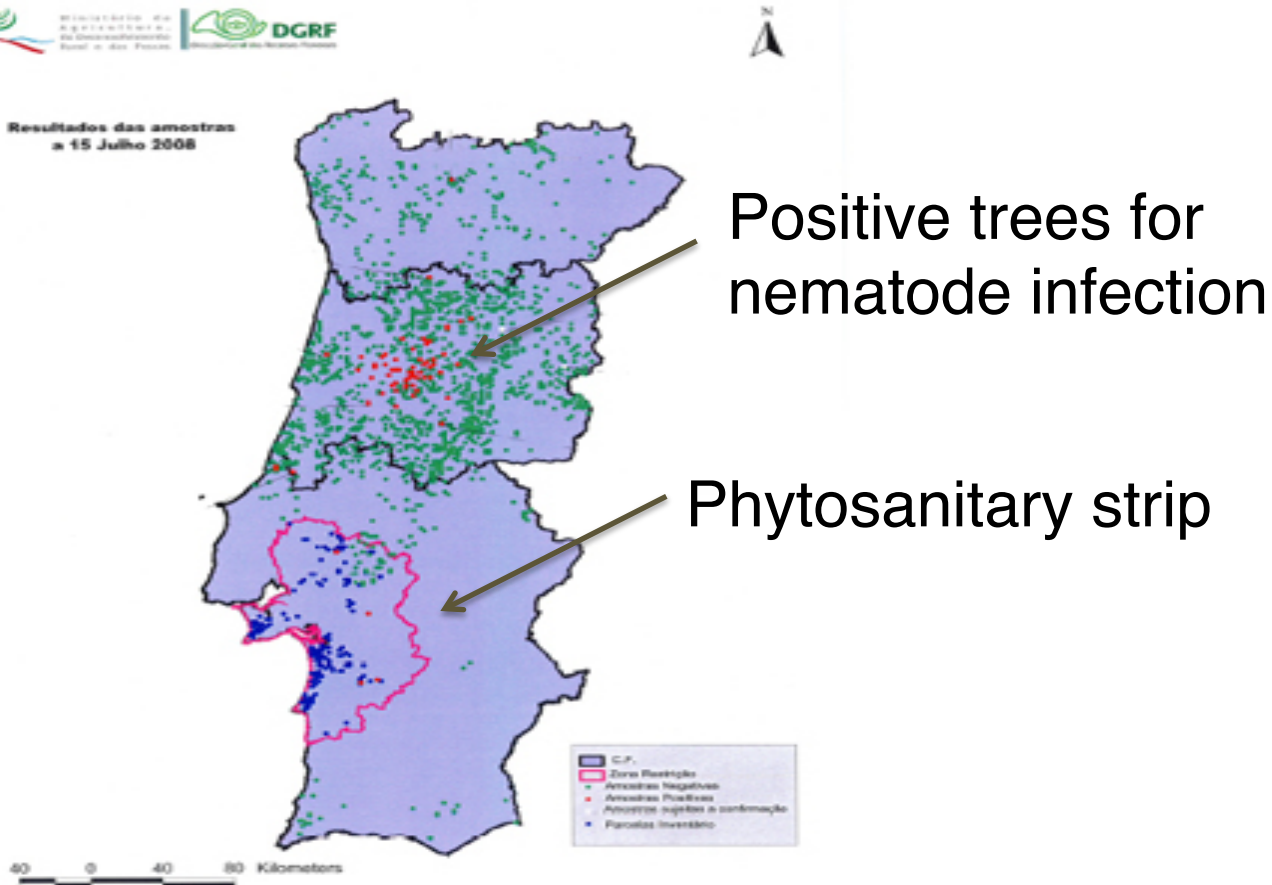
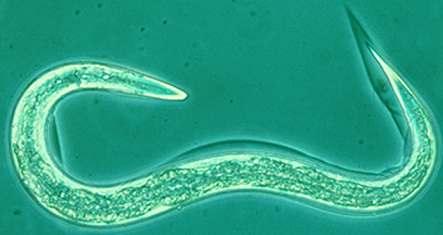
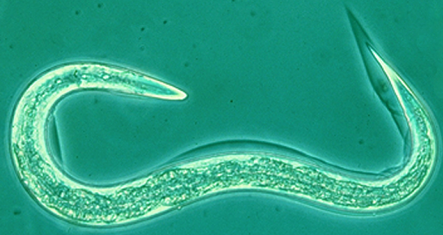


Figure 2 - *B. xylophilus* distribution in Portugal in 2008.

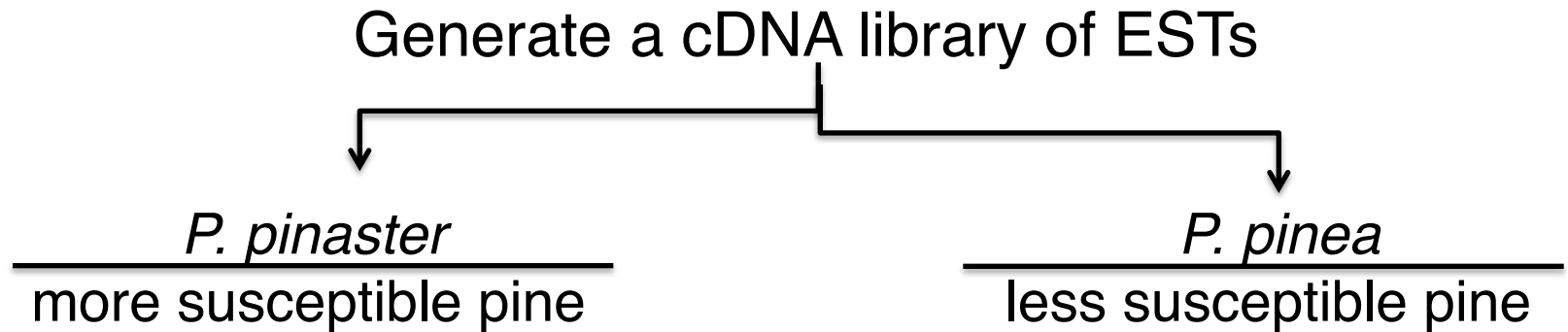


FIRST HOURS AFTER INFECTION

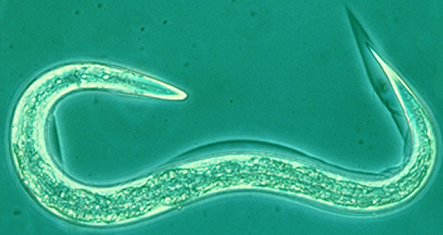
- > As the invasion by nematodes start, it is thought that an **innate hypersensitive defense mechanism** is triggered. Recent studies point out that basal defence mechanisms against pathogens occur within the first couple of hours after infection
- > This hypersensitive reaction results in the
 - release of phenolics
 - synthesis of toxins and phytoalexins
 - compartmentalization of xylem and other tissues
 - flooding of tracheids with oleoresin and toxic substances
- > **HR** is thought to be activated by a genetic program, where resistance genes recognize certain effectors thus initiating a resistance response that is frequently linked to a rapid cell death



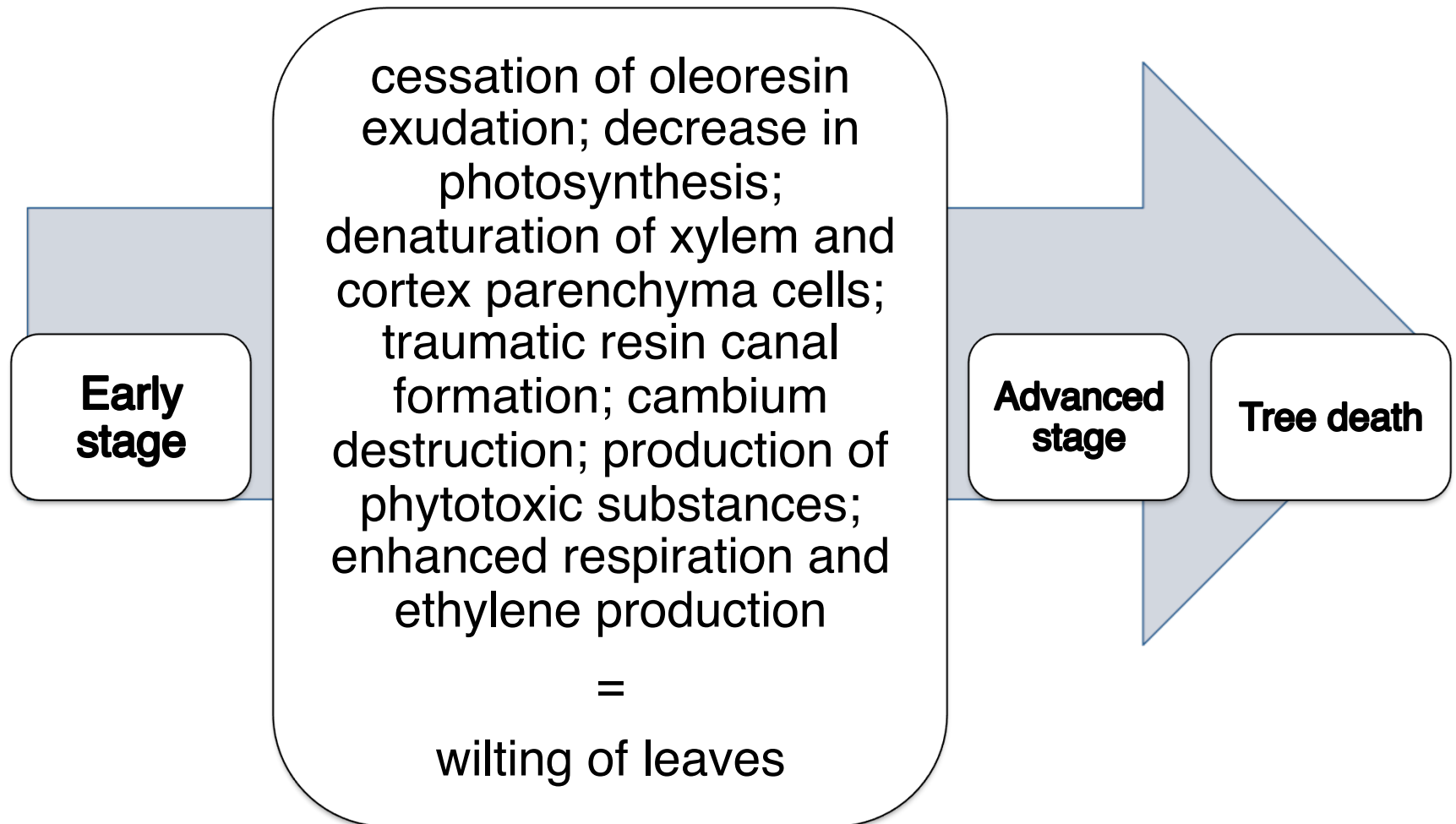
OUR GOAL

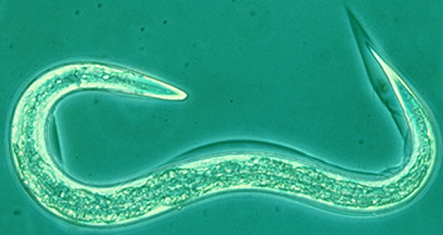


- Implementation of the SSH technique for the study of pine differentially expressed genes;
- Cloning of differentially expressed genes between *P. pinea* and *P. pinaster*-inoculated plants using the SSH technique.



Symptom development is the only tool that allows an observer to identify a diseased tree





METHODS

Nematode culture

26 °C – 7 d

26 °C – 7 d

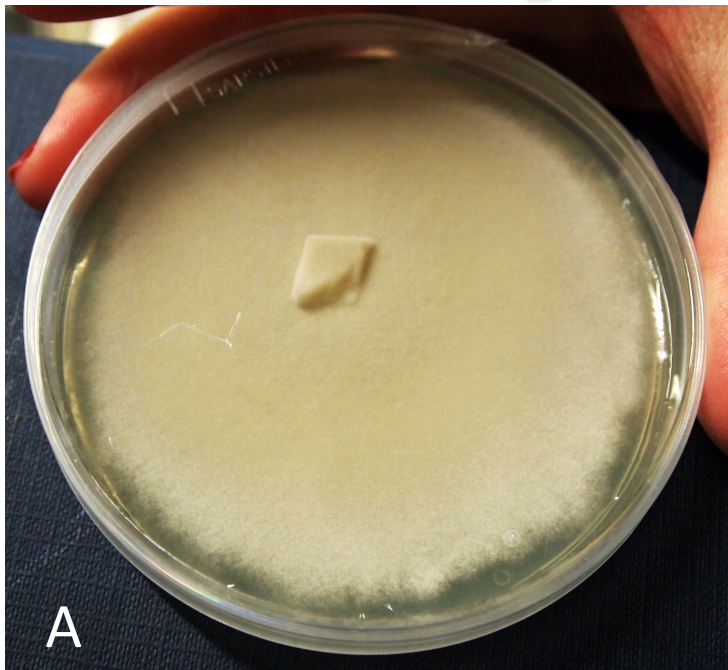
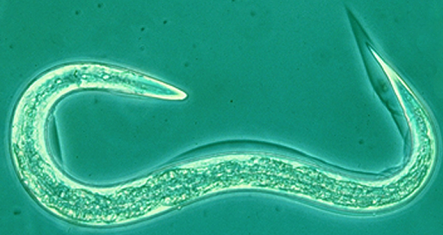
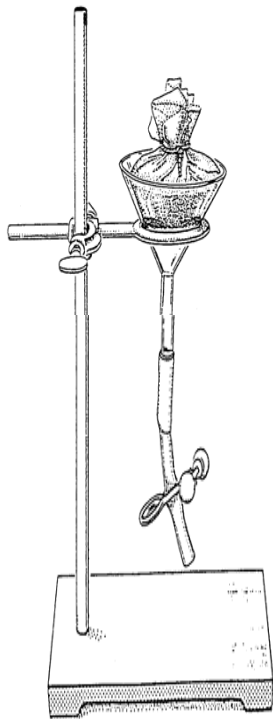


Figure 3 – A) *Botrytis cinerea* cultured in PDA; B) *Botrytis cinerea* cultured in barley grains; C) *Bursaphelenchus xylophilus* strain HF.



METHODS

Inoculation



> 4 *P. pinea* + 4 *P. pinaster* inoculated with a suspension with 1000 *B. xylophilus* strain HF

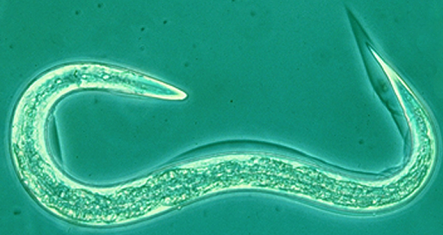
3 hours incubation

RNA extraction

cDNA synthesis

Suppression Subtractive Hybridization

Figure 4 - Baermann funnel technique (Baermann, 1917)



METHODS

Diatchenko *et al.* (1996) described the method of suppression subtractive hybridization (SSH):

> identify and isolate cDNA fragments differentially expressed

> can selectively suppress amplification of undesirable sequences in PCR procedures

> hybridization step: normalizes sequence abundance during the course of subtraction, enriching only the differentially expressed cDNAs.

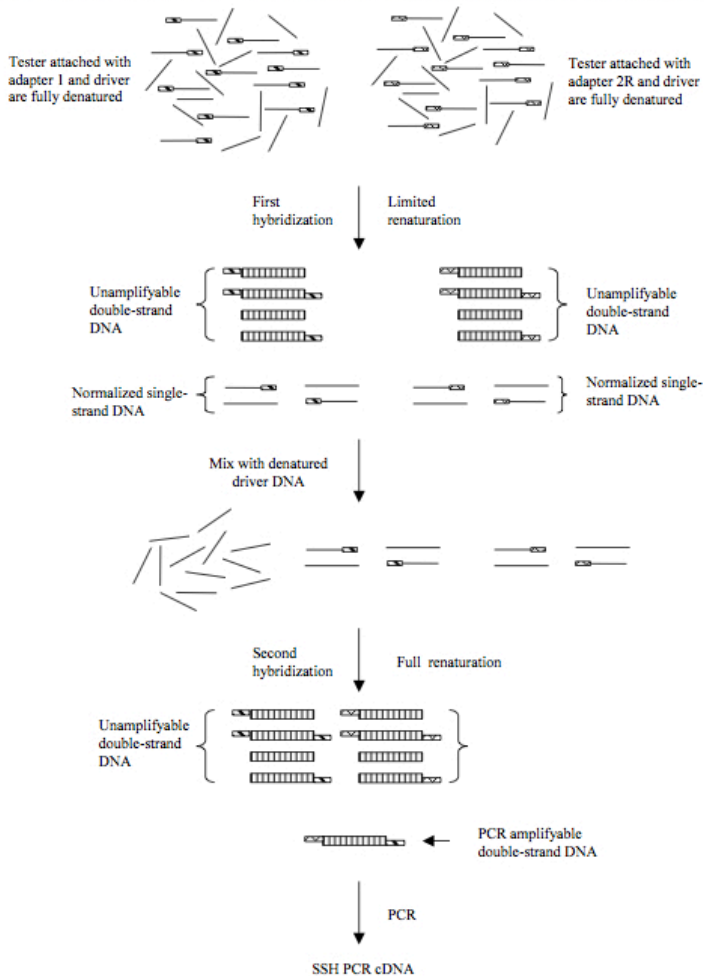
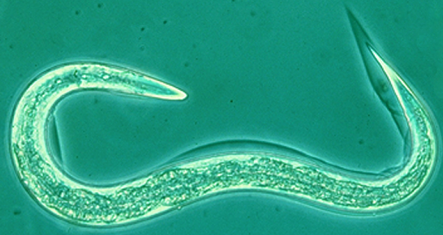


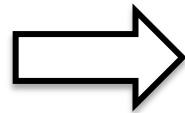
Figure 5 – Scheme of the SSH method (from Ji *et al.*, 2002).



RESULTS

Four different libraries

A & B: Differentially expressed genes



A

Forward
subtraction

P. pinaster+HF

B

Reverse
subtraction

P. pinea+HF

C

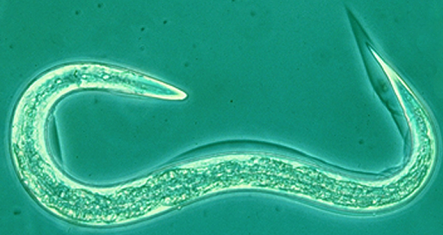
Unsubtracted
Tester:

P. pinaster+HF

D

Unsubtracted
Driver:

P. pinea+HF



RESULTS

Forward Subtraction



12 sequences



No homology in BlastN and
BlastX searches of NCBI

Reverse Subtraction



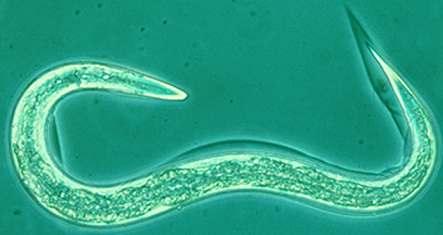
33 sequences



5 had hits in BlastN and
BlastX searches of NCBI



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



RESULTS

Forward Subtraction



12 sequences



No homology in BlastN and
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Reverse Subtraction



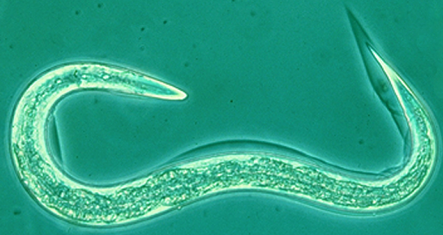
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5 had hits in BlastN and
BlastX searches of NCBI



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*?



RESULTS

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

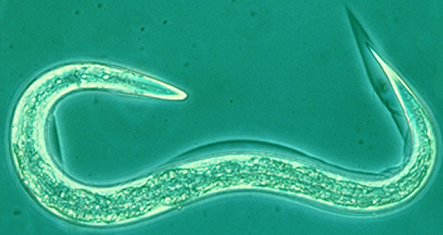
Possible phenylalanine ammonia lyase

Non-specific lipid-transfer protein type 1

Hypothetical xyloglucan endotransglycosylase

Genes of (or related to) RNA recognition motif

oxidative stress related



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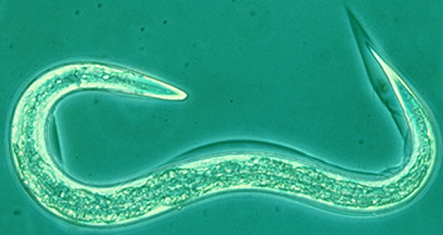
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Non-specific lipid-transfer protein type 1

Hypothetical xyloglucan endotransglycosylase

Genes of (or related to) RNA recognition motif

defence related



RESULTS

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

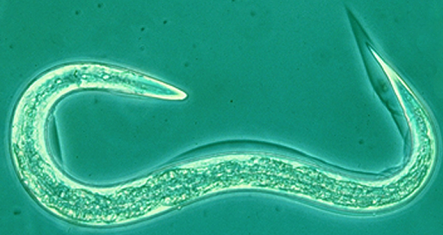
Probable RNA recognition motif

Sm-like protein

Protein similar to one belonging to DUF231 *Arabidopsis* proteins

NifU-like protein

defence related



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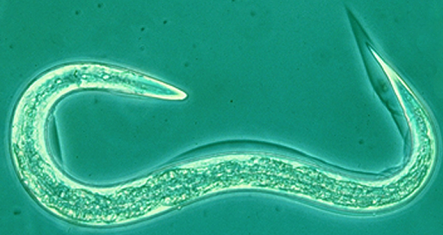
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ethylene biosynthesis



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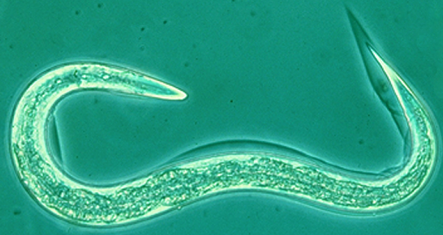
Probable RNA recognition motif

Sm-like protein

Protein similar to one belonging to DUF231 *Arabidopsis* proteins

NifU-like protein

RNA processing events



GENE EXPRESSION CONFIRMATION

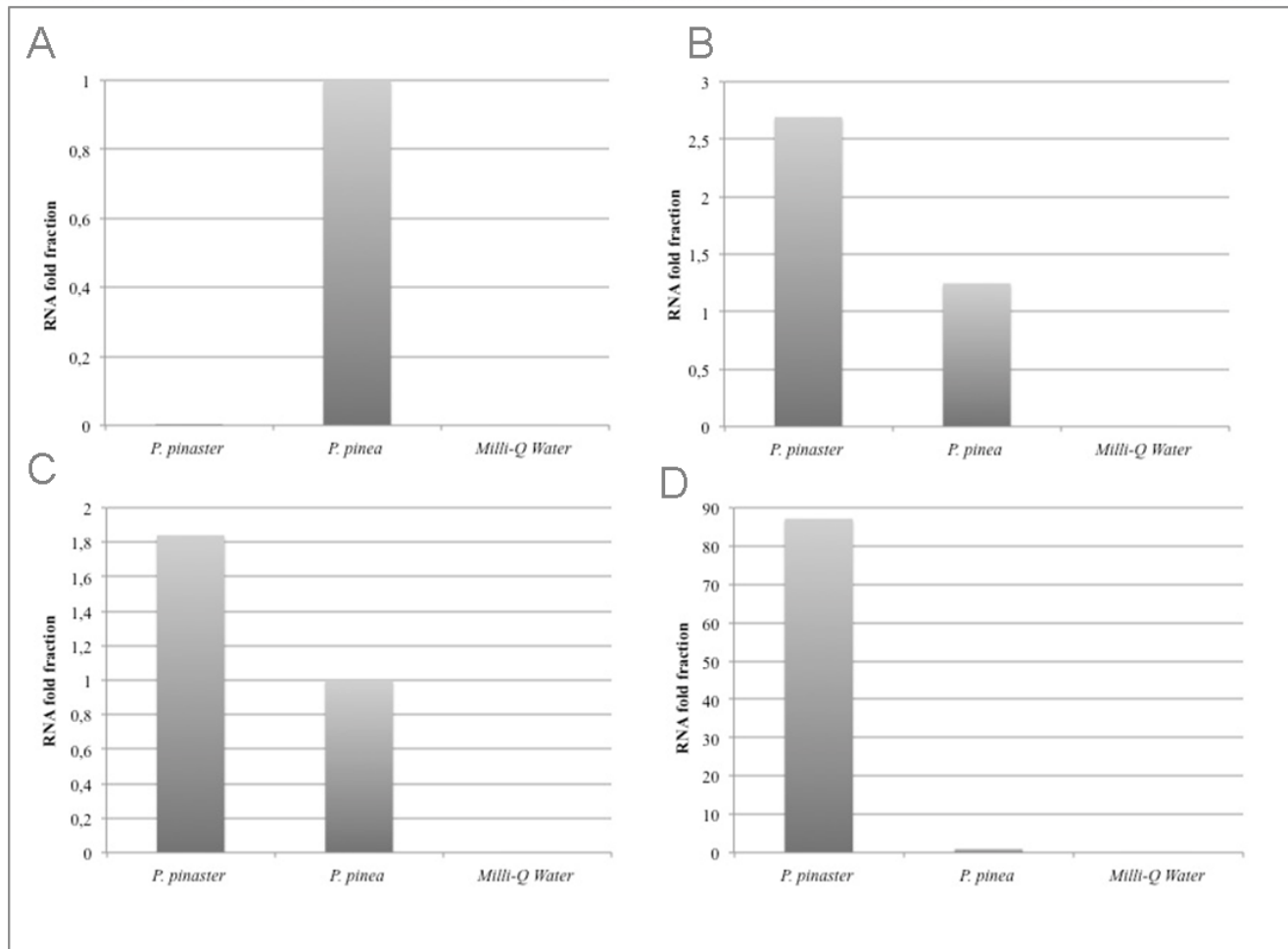
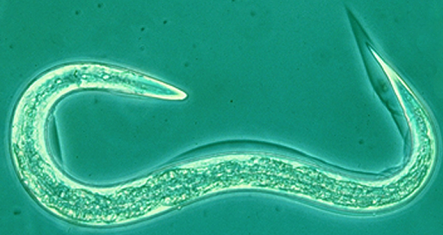
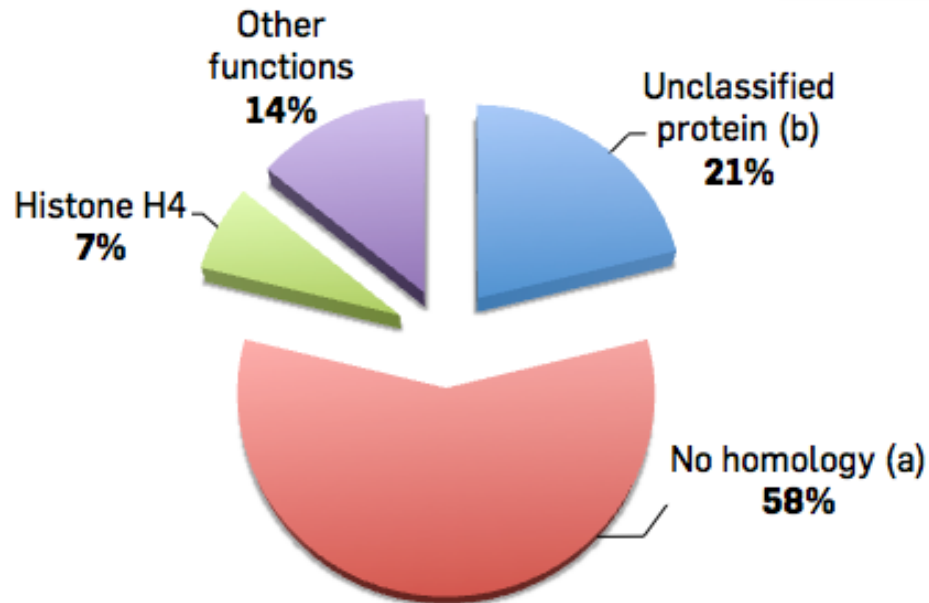


Figure 6 - A) Histone H4 B) *P. pinaster* Unknown C) *P. pinaster* drought stress D) PAL



FUNCTIONAL CATEGORIZATION

A) Functional categorization of induced genes.



B) Conserved domain information about the 15 genes annotated as “unknown protein” in NCBI databases.

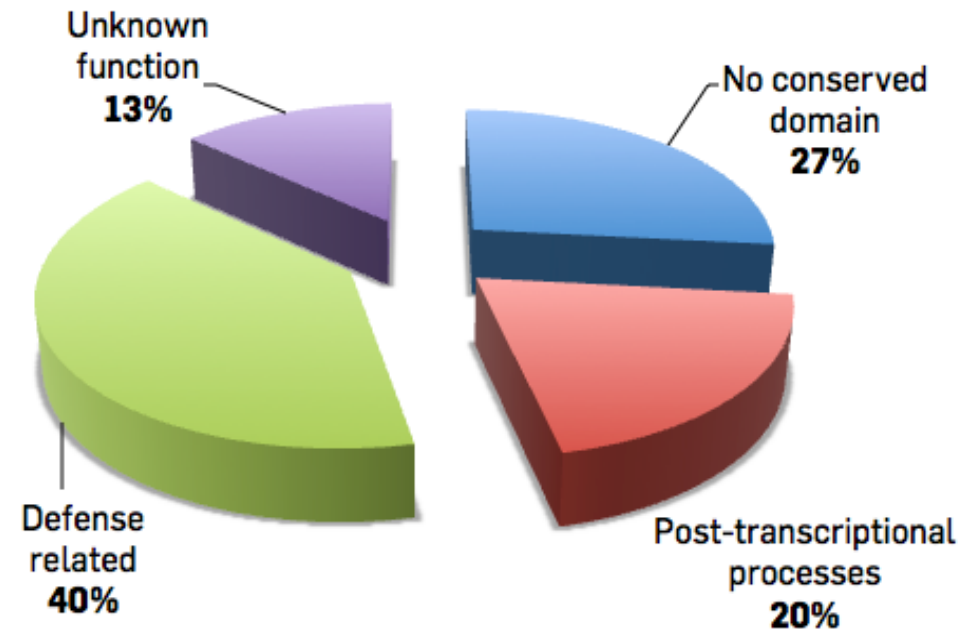
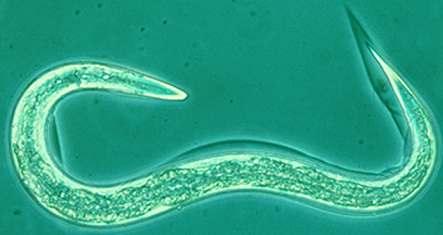


Figure 7 - All 71 genes were functionally annotated using BlastN and BlastX at NCBI, and categorized based on GO annotation. (a) These genes had no significant hits in BlastN and BlastX searches of NCBI. (b) These genes matched proteins annotated as ‘unknown protein’.



GENUS CATEGORIZATION

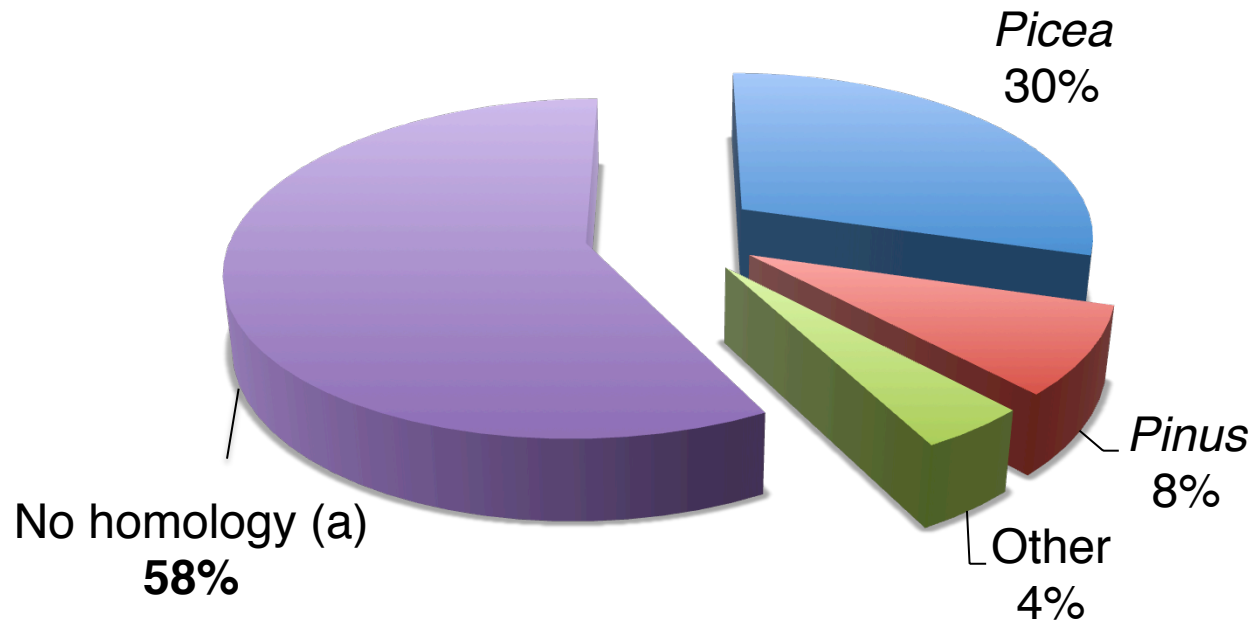
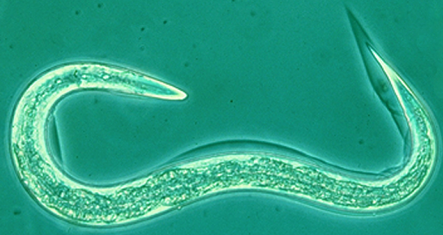


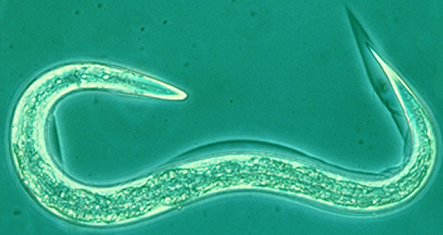
Figure 8 – Genus characterization of induced genes.

(a) These genes had no significant hits in BlastN and BlastX searches of NCBI.



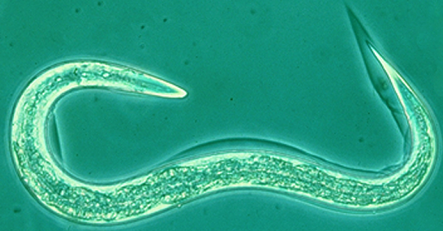
CONCLUSIONS

- **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 defence mechanisms;
- **Oxidative stress** was found to be a very important defence 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;

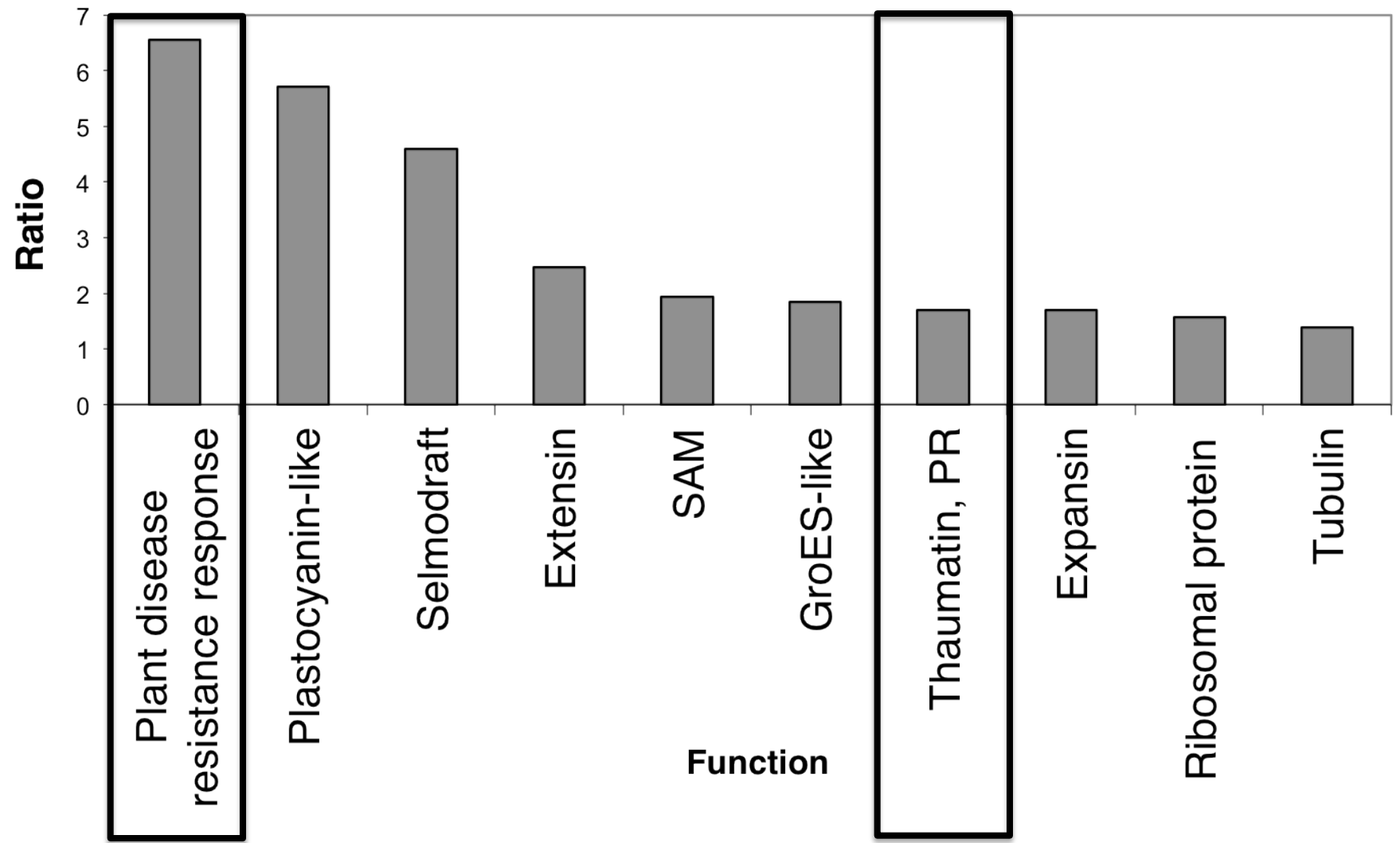


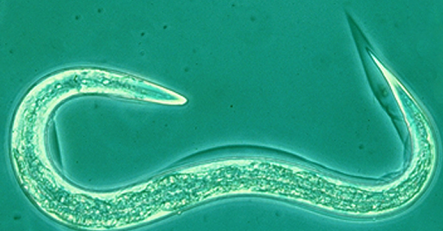
FUTURE WORK

- **Pyrosequencing** is under course to better understand pine transcriptome under infection with PWN
 - > A preliminary analysis of the results is being conducted
 - > A total of **1,423,649** sequences were obtained
 - > Differential expression of basal defence genes was identified

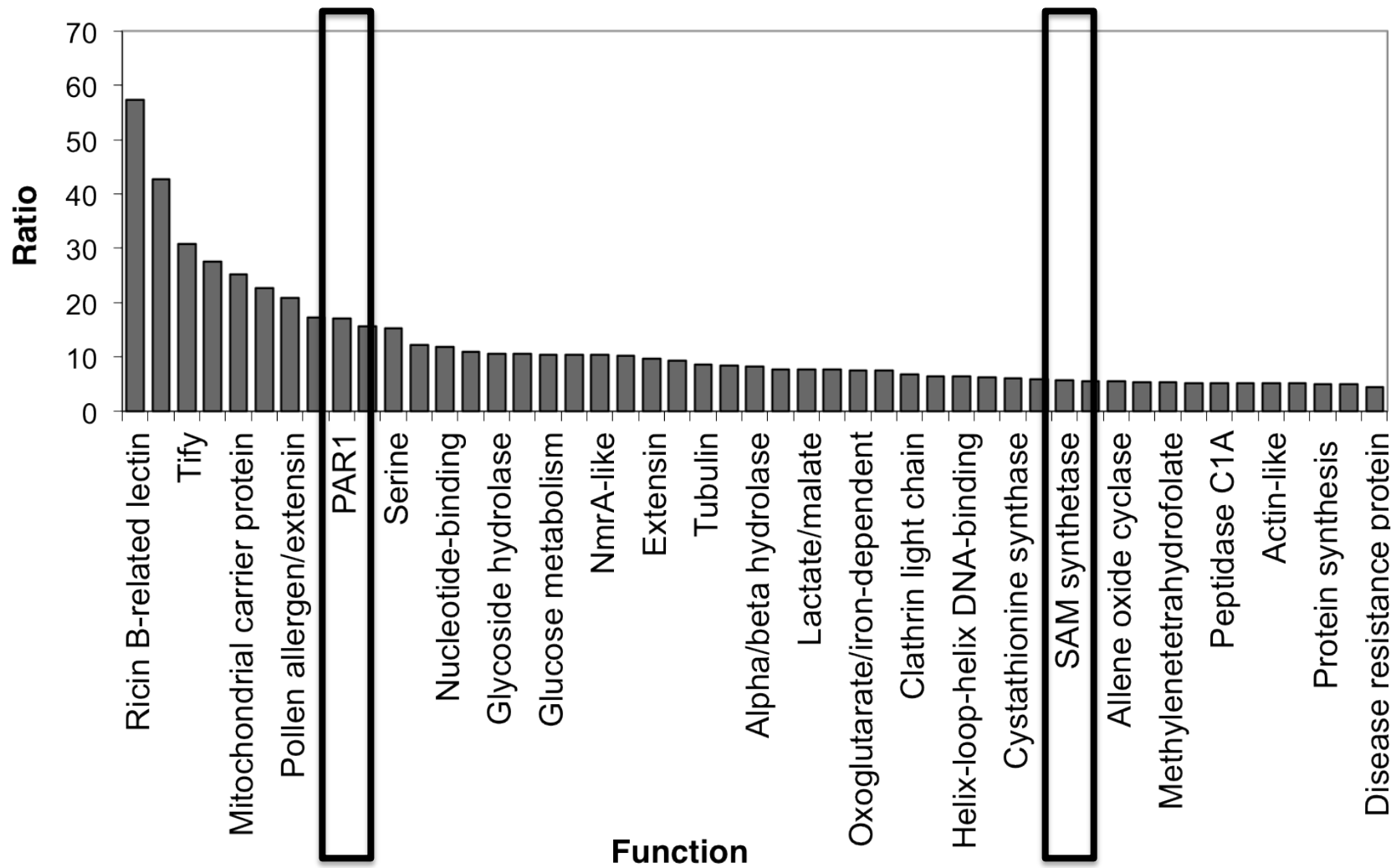


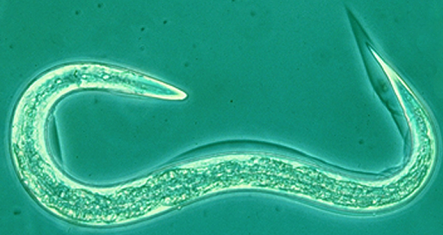
UP-REGULATED GENES IN *P. pinaster*+PWN VS. CONTROL





UP-REGULATED GENES IN *P. pinea*+PWN VS. CONTROL





ACKNOWLEDGMENTS

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**THANK YOU FOR YOUR
ATTENTION**