FUNCTIONAL GENOMIC APPROACHES TO ELUCIDATE BIOTIC AND ABIOTIC STRESS RESPONSES IN PLANTS

PhD Course in Agricultural, Forestry and Environmental Science (XXIX ciclo)

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INTRODUCTION

ABIOTIC STRESS

(Drought, Salinity, Extreme temperature, Humidity)

BIOTIC STRESS

(Bacteria, Virus, Fungi, Herbivores)

G-protein, kinases, phosphatases, transcription factors, transporters, receptors

"Induced Stress Response"

Early Diagnosis of Diseases and Disorders

Gene regulations of key genes and pathways

(sucrose and starch metabolism, hormone metabolism, transcription factors)

AIM OF THIS WORK

The use of innovative functional genomics techniques to gain insight into molecular mechanisms of regulation of crop responses to biotic and abiotic stress

Biotic stress responses:

1. Large-scale analysis of the gene regulatory networks of *Phoenix canariensis* (Chabaud) in response to the attacks of *Rhynchophorus ferrugineus* (Olivier) (Contribution 1)
2. Microarray analisys in grapevine to elucidate early responses and recovery mechanisms to "stolbur" infection (Contribution 2)
3. Molecular responses to small regulating molecules against Huanglongbing disease in *Citrus* (Contribution 3)
4. Proteomic responses of two Citrus genotypes with variable tolerance to HLB, in order to identify proteins playing a key role in the diverse phenotypic sensitivity to the disease (Contribution 4)

Abiotic stress responses in durum wheat:

5. Annotation and characterization of miRNAs in response to drought stress (Contribution 5)
6. Analyze the agronomic and key molecular responses to salt stress and mycorrhizal inoculation (cv. Anco Marzio) (Contribution 6)

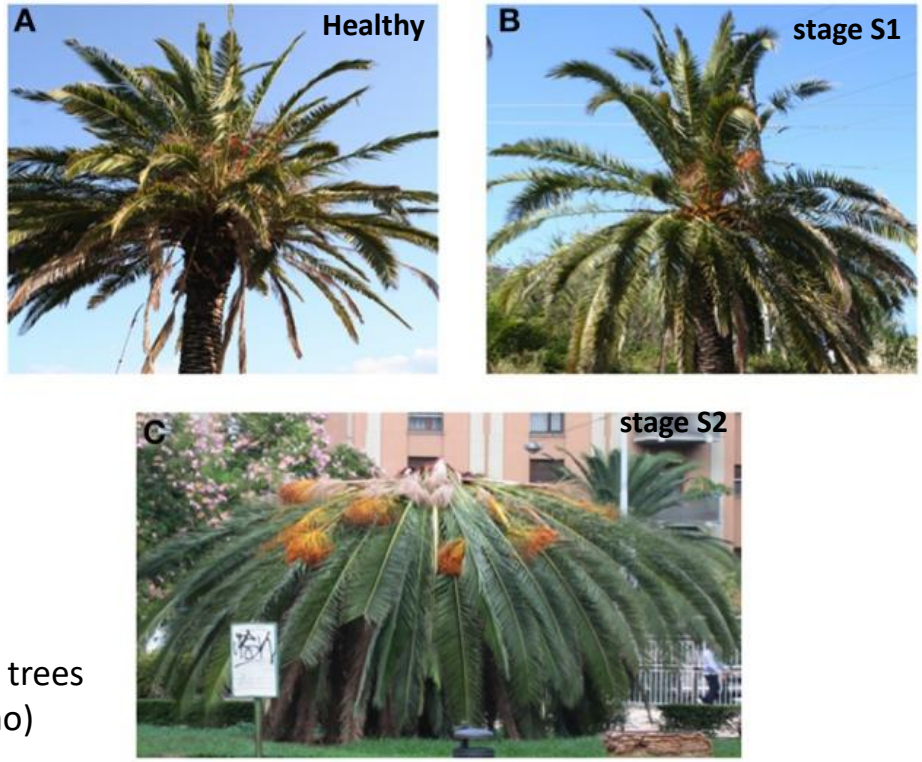
CONTRIBUTION 1

TRANSCRIPTOME ANALYSIS OF *Phoenix canariensis CHABAUD IN RESPONSE TO Rhynchophorus ferrugineus OLIVIER ATTACKS*

Aim of this study

(1) clarify the gene regulation mechanisms of leaf metabolism in response to RPW attacks at different stage of infestations

(2) identify possible host biomarkers that may confirm RPW typical symptomatology



15-20-year-old trees
(Trabia, Palermo)

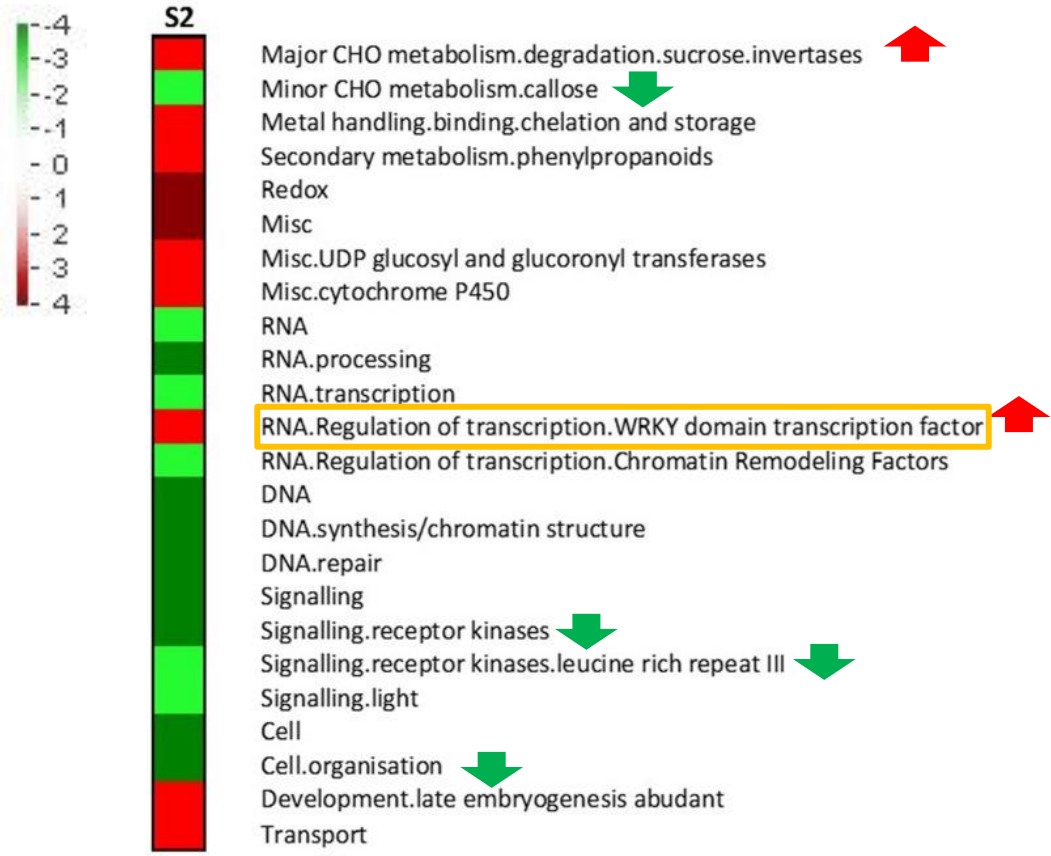
Giovino A, Bertolini E, Fileccia V, Al Hassan M, Labra M, Martinelli F (2015) - Front Plant Sci 6:817.

PathExpress analysis of the up and downregulated genes in each of the two pairwise comparisons

S1 vs. He	*P*-values
UPREGULATED	
Phenylalaninemetabolism	4.1×10^{-3}
Phenylpropanoidbiosynthesis	5.1×10^{-3}
Flavonoidbiosynthesis	7.9×10^{-3}
Metabolism of xenobiotics by cytochrome P450	2.6×10^{-2}
DOWNREGULATED	
Flavone and flavonolbiosynthesis	1.3×10^{-2}
S2 vs. He	***P*-values**
UPREGULATED	
Fatty acid metabolism	3.3×10^{-4}
Sphingolipid metabolism	3.6×10^{-2}
Glycerolipid metabolism	3.7×10^{-2}
Tryptophan metabolism	0.05
Alkaloid biosynthesis	0.05
DOWNREGULATED	
Starch and sucrosemetabolism	2.1×10^{-3}
Phosphatidylinositol signaling system	3.6×10^{-3}
Inositol phosphate metabolism	9.3×10^{-3}
Glycosaminoglycan degradation	0.04

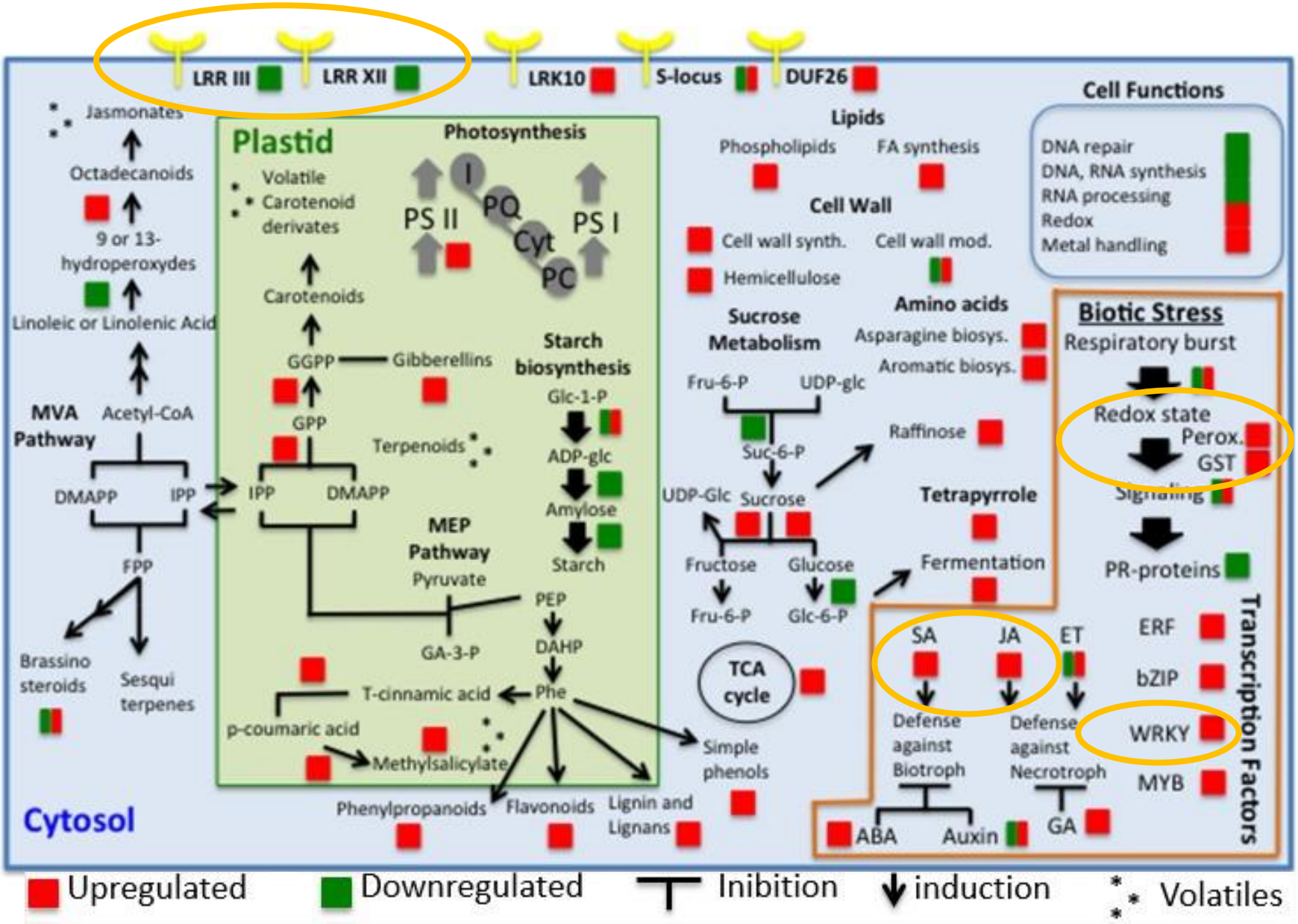
Pathways with P < 0.05 (no corrections) were affected by the RPW attacks

Gene set enrichment analysis of the transcriptomic changes during stage 2 (S2)



Pageman web-tool was used. Wilcoxon test with ORA cut off = 1 was used. A scale bar between -4 and 4 was chosen. Increased intensity of red and green respectively represented higher level of upregulation and downregulation

Global view of the transcriptomic changes in palm leaves in response to RPW attacks

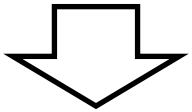


CONCLUSIONS

The ability to rapidly identify RPW infestations is of particular interest because it would allow to rapidly activate the management practices against RPW

Upregulation of some important **WRKY genes**

Key players involved in transcription regulation of biotic stress responses



They might be considered candidate host biomarkers for RPW infestations

CONTRIBUTION 2

A MICROARRAY ANALYSIS HIGHLIGHTS THE ROLE OF TETRAPYRROLE PATHWAYS IN GRAPEVINE RESPONSES TO "STOLBUR" PHYTOPLASMA, PHLOEM VIRUS INFECTIONS AND RECOVERED STATUS

Aim of this study

to study the global transcriptomic changes for grapevine leaf responses to "stolbur" infection to identify host biomarkers for the characterization of the following stages:

(1) Asymptomatic stage – Phy AS (2 replicates)

(2) Symptomatic stage – Phy SY (2 replicates)

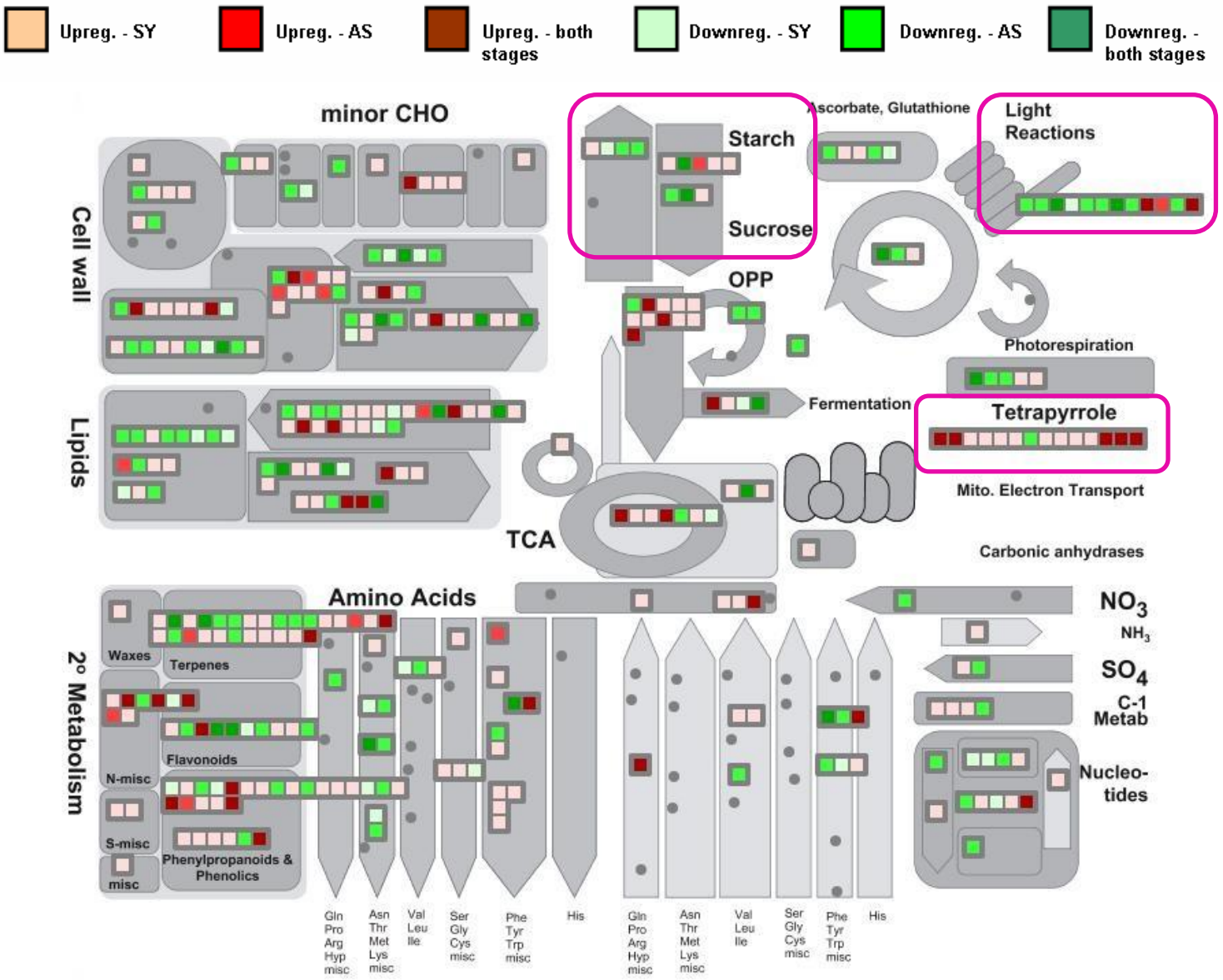
(3) Coinfected with viruses – Phy + virus (GLRaV-3 and GVA viruses) (2 replicates)

(4) Recovered stage – Re (2 replicates)

Symptoms on «bois noir»-infected 'Montepulciano' plants: reddish discoloration and downwardly rolled margins of leaves. (Giulianello, Latina)

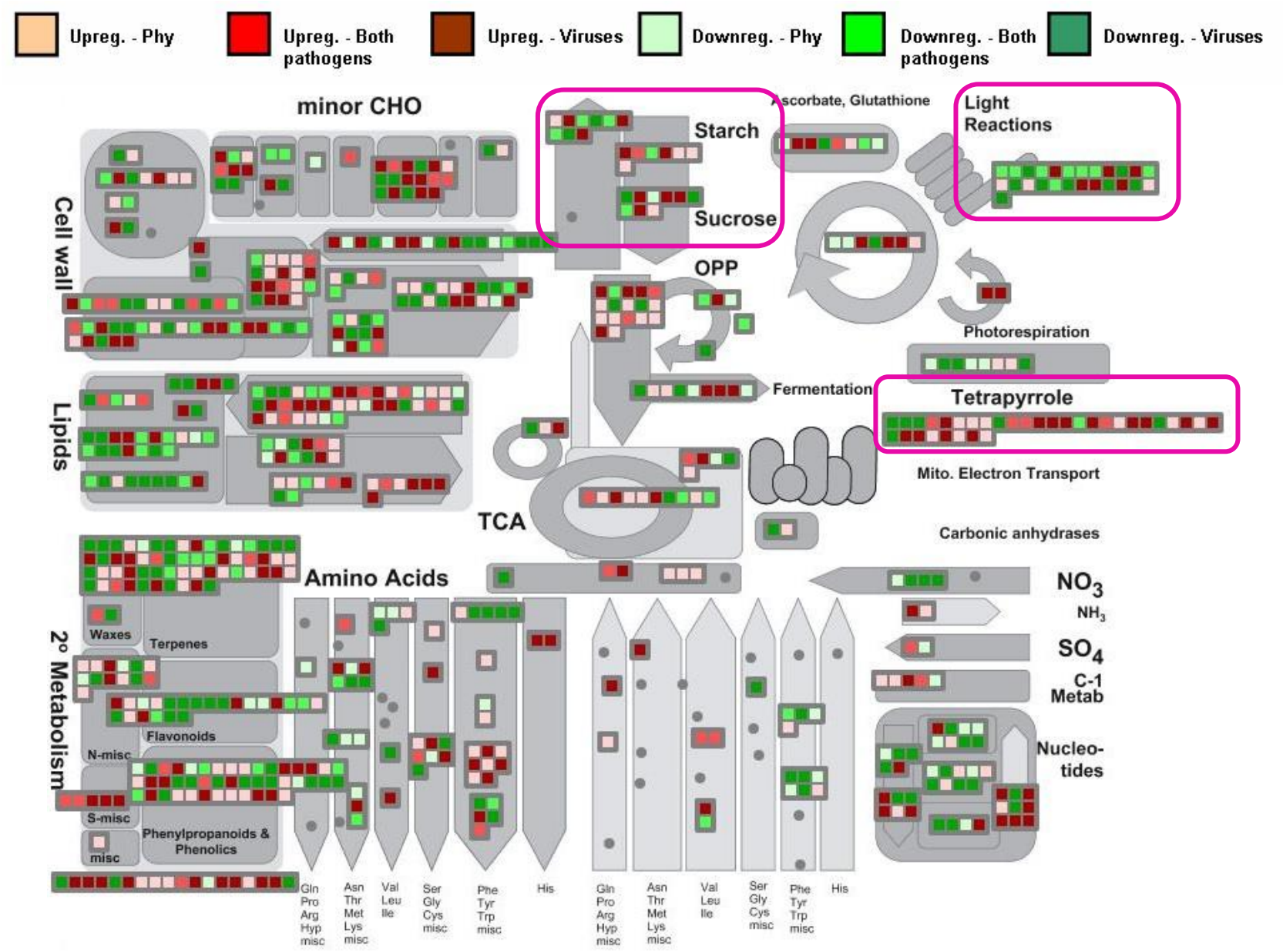
Punelli F, Al Hassan M, Fileccia V, Uva P, Pasquini G, Martinelli F (2016) - Physiol Mol Plant Pathol 93:129-137.

GRAPEVINE RESPONSES TO ‘STOLBUR’ INFECTION



PRIMARY METABOLISM OF RECOVERED STATUS

GRAPEVINE RESPONSES TO COINFECTION WITH VIRUSES



CONCLUSIONS

A determinant role in symptom appearance and in plant decline is played by imbalances in molecular transport through the phloem system, which then cause sink-source disorders

This work identified important players in this network

Genes involved in **tetrapyrrole pathways**

Can contribute to develop early disease diagnostics
and new control strategies

These findings will help to uncover disease mechanisms and to facilitate the development of early diagnostic tools and short-term control strategies

CONTRIBUTION 3

MOLECULAR RESPONSES TO SMALL REGULATING MOLECULES AGAINST HUANGLONGBING DISEASE

Aim of this study

to determine if small molecules are effective in modulating expression of key HLB-regulated or innate response Citrus genes after three to six days of treatment

HLB disease is caused by the phloem-specific CaLas vectored by ACP

Symptoms: blotchy mottled leaves and yellow shoots, stunted trees, declining, few, small-sized, and deformed poorly colored fruits.

Diagnosis: Based on symptoms and pathogen confirmation with RT-PCR.

Management: Tree eradication and pesticide sprays to control ACP

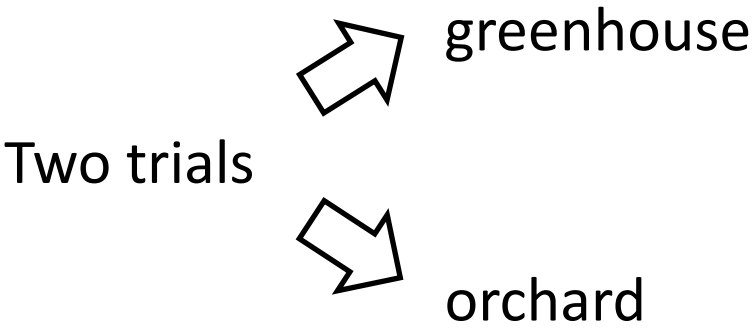
Martinelli F, Dolan D, Fileccia V, Reagan RL, Phu M, Spann TM, Mccollum GT, Dandekar A (2016) - PLoS ONE 11(7):e0159610.

We tested three small-molecule modulating *Citrus* molecular response to HLB disease beneficially (2 different concentrations):

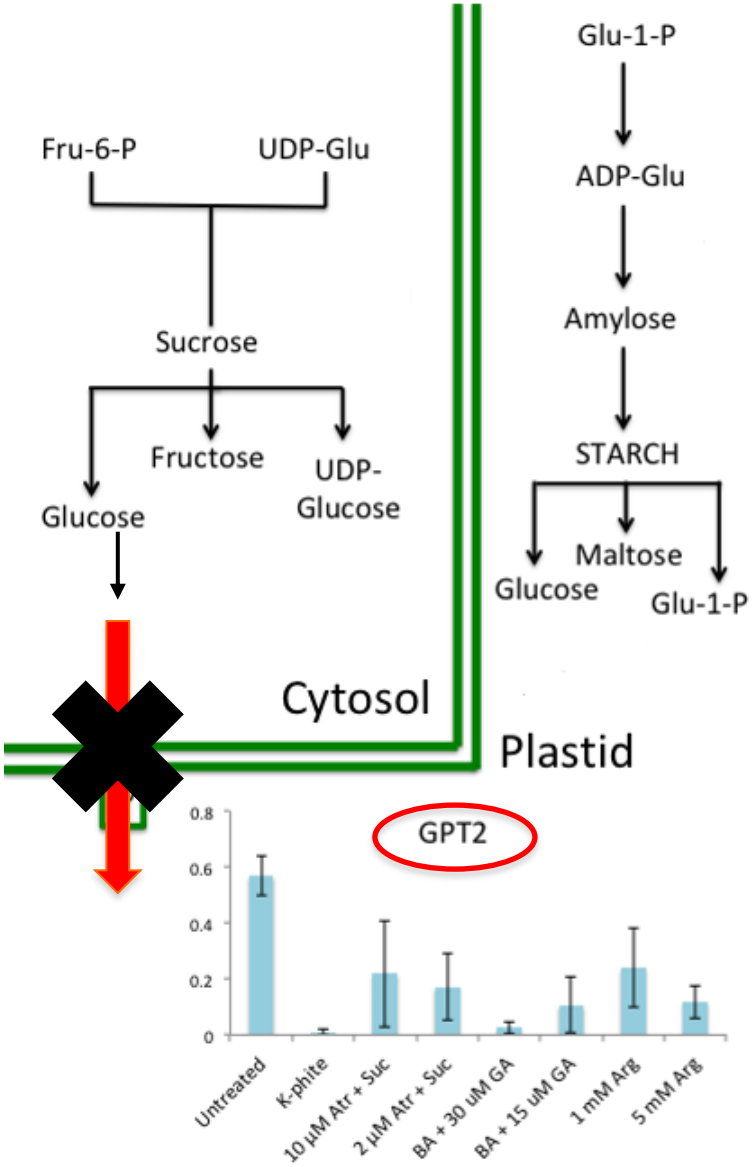
1) **L-Arginine** to boost SAR response against *Candidatus liberibacter asiaticus*

2) **6-benzyl-adenine** combined with **gibberellins** to downregulate starch biosynthesis and upregulate its degradation

3) **Sucrose** combined with **atrazine** to induce xenobiotic signaling and ROS signaling

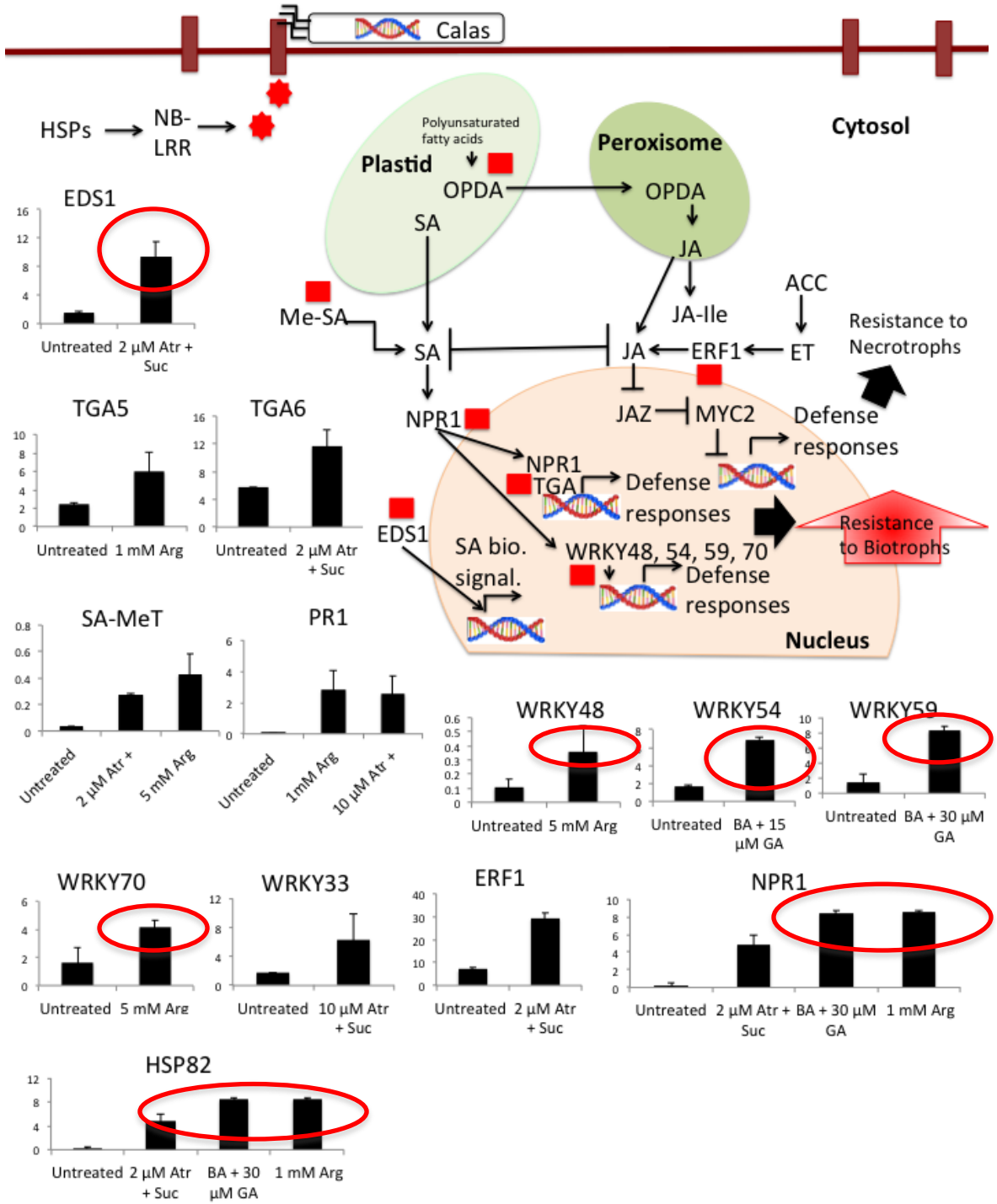


TREATMENT EFFECTS ON SUGAR AND STARCH METABOLISM



Hypothesis

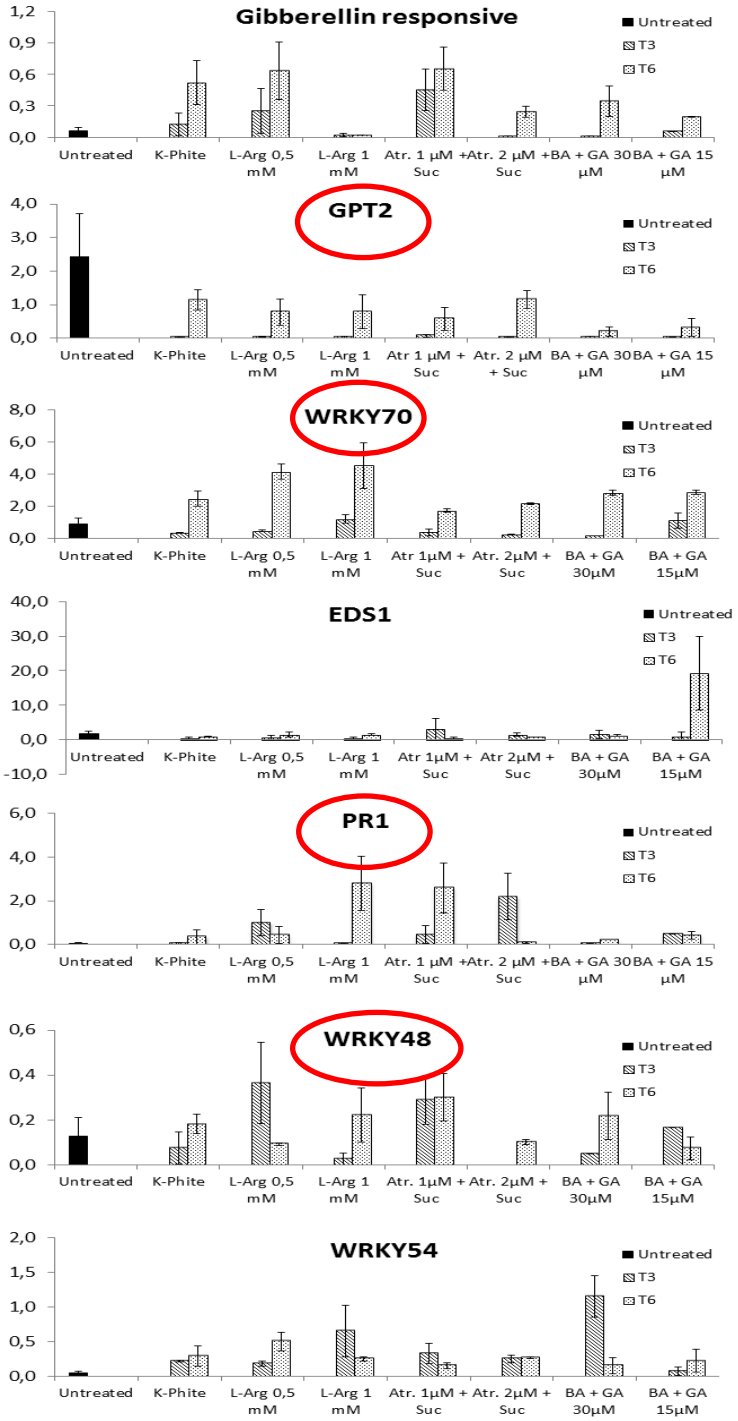
The Downregulation of GPT2 by all six treatments may decrease the import of glucose in the chloroplast and decrease starch accumulation caused by HLB disease



TREATMENT EFFECTS ON BIOTIC STRESS RESPONSES

Hypothesis

The upregulation of key genes involved in salicylic acid signaling (NPR1,EDS1, WRKYs) may be of benefit for the host in early stages of infection



FIELD TRIAL

Seven Host Biomarkers were selected to analyze the responses to treatments in field conditions at 3 and 6 days after infection

Main Results:

1) GPT2 was confirmed to be downregulated

2) WRKY48, WRKY70 and PR1 were mostly upregulated by arginine treatments

Conclusions

The small-molecule sprays may affect transcript abundance of key genes involved in HLB carbohydrate metabolic syndrome and innate responses

Future studies should examine long-term application of treatments that combine these molecules in field trials

CONTRIBUTION 4

PROTEOMIC ANALYSIS HIGHLIGHTS THE ROLE OF DETOXIFICATION PATHWAYS IN INCREASED TOLERANCE TO HUANGLONGBING DISEASE

Aim of this study

to identify key proteins involved in the diverse susceptibility mechanisms of Huanglongbing disease

"Blotchy mottle symptoms" associated with HLB

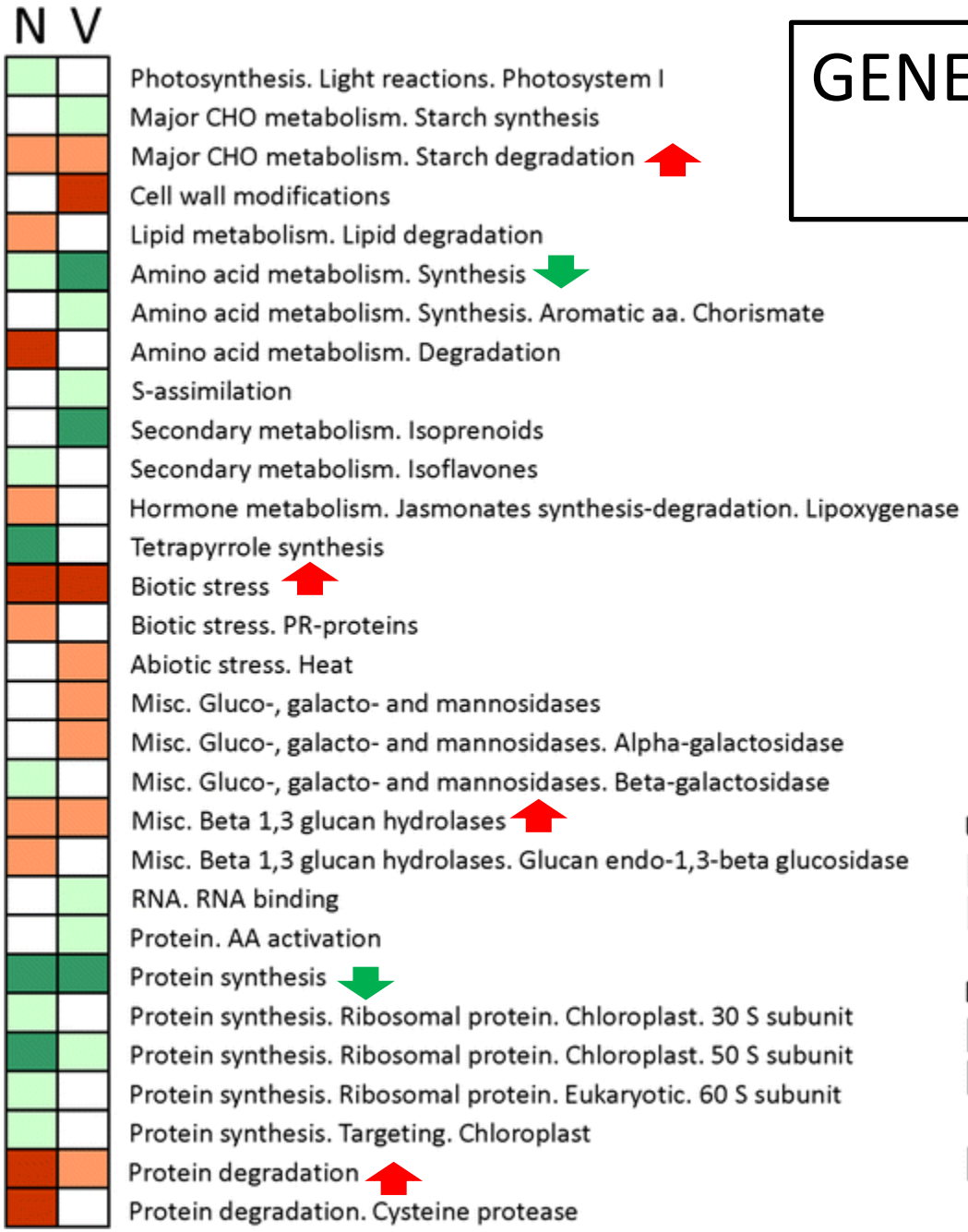
Two *Citrus* genotypes:

1) **Navel Orange (N)** (*Citrus sinensis*): HLB-sensitive cultivar
2) **Volkameriana (V)** (*Citrus volkameriana*): HLB moderately tolerant

Four categories of samples: (1) Healthy V
(2) Healthy N
(3) Infected V
(4) Infected N

Martinelli F, Reagan RL, Dolan D, Fileccia V, Dandekar A (2016) - BMC Plant Biology 16:167.

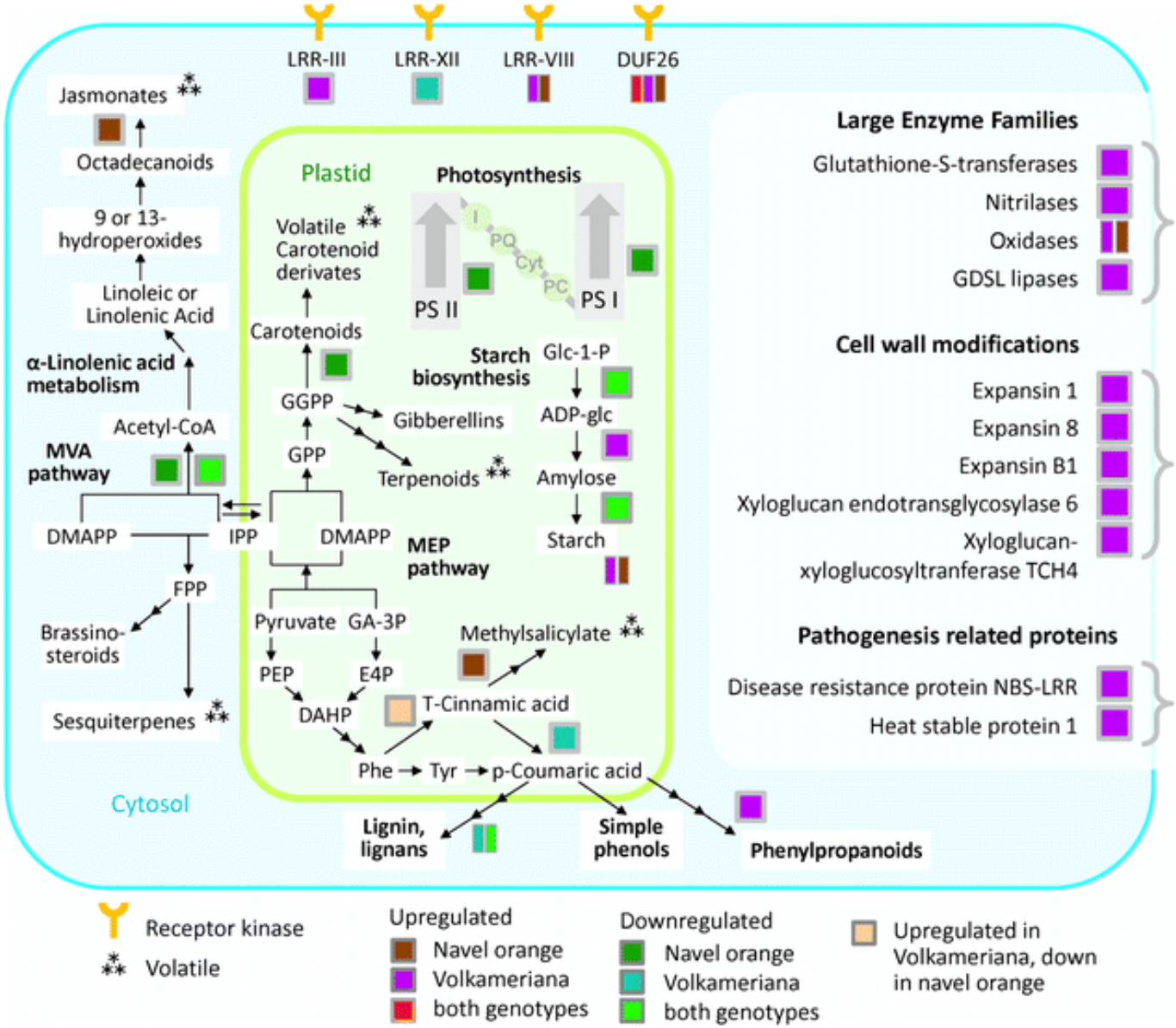
GENE SET ENRICHMENT ANALYSIS



Upregulated in infected samples
- Log fold ratio ≥ 3
- Log fold ratio > 0.5 and < 3

Downregulated in infected samples
- Log fold ratio ≤ −3
- Log fold ratio < −0.5 and > −3

- Not significantly regulated in infected samples



Upregulation in infected V and not in infected N explain the increased tolerance of Volkameriana compared to Navel orange

GLOBAL VIEW OF PROTEOMIC CHANGES IN CITRUS LEAVES (NAVEL ORANGE AND VOLKAMERIANA) IN RESPONSE TO HLB INFECTION

Conclusions

An integrated approach using PCA, gene set enrichment analysis and functional data was applied to identify specific key proteomic changes in response to Huanglongbing disease in these two *Citrus* genotypes.

The clearest differences were observed for proteins involved in detoxification pathways such as **glutathione-S-transferases**, **nitrilases** and **GDSL lipases**

CONTRIBUTION 5

IDENTIFICATION AND CHARACTERIZATION OF DURUM WHEAT MicroRNAs IN LEAF AND ROOT TISSUES

Aim of this study

to annotate and map miRNA in relation to their expression in leaf and root tissues under two conditions: (1) irrigated to 100% and (2) 55% of evapotranspiration (early water stress)

Cultivar Simeto: sensitive to drought (Bresta et al. 2011)

4 different water regimes:

Contr100: total replenishment of lost water daily

STR85
STR70
STR55

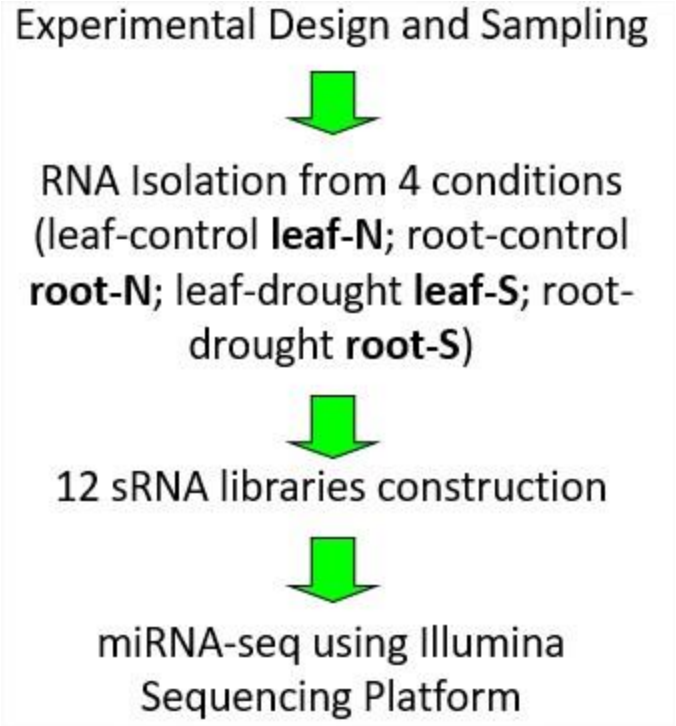
Replenishing of 85%, 70% and 55% of the daily evapotranspiration, respectively, as measured on Contr100

The extreme water stress treatment were chosen for the characterization of durum wheat miRNAome

The pots were weighted daily and the water amounts were regulated by weight

All pots were harvested after 10 days from the start of water stress

Fileccia V, Bertolini E, Ruisi P, Giambalvo D, Frenda AS, Cannarozzi G, Tadele Z, Crosatti C, Martinelli F (2016) - In press to Functional & Integrative Genomics



66.795 small RNA clusters

90 high confidence miRNA loci were found

58 were found to be conserved in both leaves and roots (absent in 3 chromosomes)

1/3 were classified as putative novel miRNAs (distributed among the 7 chromosome)

	Roots		Leaves	
	Control	**Drought**	**Control**	**Drought**
Total raw reads	58.677.892	50.893.655	30.665.332	48.898.744
Uniquely mapped reads	6.789.788	4.963.645	2.404.013	3.487.512
Multi mapped reads	20.549.748	16.102.052	21.173.031	37.276.683
Total mapped reads	27.339.536 (46%)	21.065.697 (41%)	23.577.044 (76%)	40.764.195 (83%)

The average number of reads for the three biological replicates in each condition and tissue are shown

HOMEOLOGOUS IDENTIFICATION

the 58 miRNAs expressed in both leaves and root tissues were mapped to the A and B genomes

7 miRNA groups within homeologous chromosomes

Chromosome mapping of homeologous identified miRNA

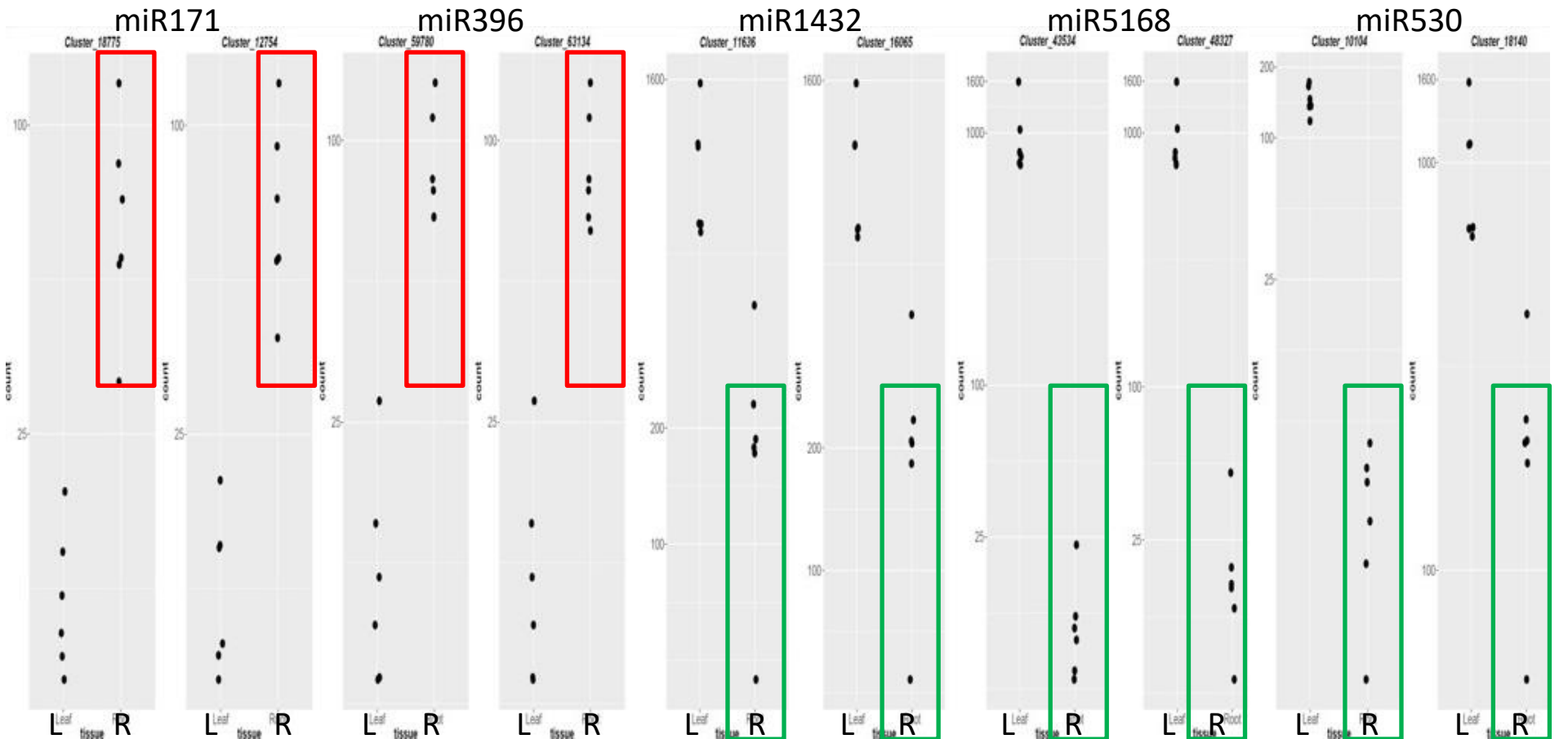
Cluster name	Chromosome	Coordinates(Start-End)	Strand	miRNA type
Cluster_59780	7A	20932743-20932904	+	ata-MIR396e
Cluster_63134	7B	8739076-8739320	-	ata-MIR396e
Cluster_18775	2B	259602537-259602631	+	ata-MIR171a
Cluster_12754	2A	210529750-210529991	-	ata-MIR171a
Cluster_53245	6A	87949906-87950062	+	bdi-MIR156c
Cluster_57238	6B	96378404-96378579	+	bdi-MIR156c
Cluster_10104	2A	45376842-45377015	+	tae-MIR530
Cluster_18140	2B	217777285-217777488	+	tae-MIR530
Cluster_43534	5A	73069415-73069551	-	ata-MIR5168
Cluster_48327	5B	163330209-163330340	+	ata-MIR5168
Cluster_11636	2A	143414592-143414715	-	ata-MIR1432
Cluster_16065	2B	84759598-84759722	-	ata-MIR1432
Cluster_43357	5A	61589422-61589539	+	tae-MIR156
Cluster_48425	5B	168674312-168674427	-	tae-MIR156

DIFFERENTIAL EXPRESSION ANALYSIS

Differential miRNA expression profiles were observed between leaf and root tissues (but not between the conditions drought vs control)

A total of 45 miRNAs were differentially expressed between root and leaf (**23** miRNAs induced in root such as miR166, **miR171**, miR319, miR167; **22** miRNAs induced in leaf such as **miR530**, miR395, **miR393**, **miR5168**, **miR396**)

Due to the high variance between the 3 biological replicates (Control leaf where found more similar to the water stressed treatment)



Conclusions

1. This work represents one of the genome wide characterization of *MIR* genes in durum wheat, identifying leaf and root tissue-specific microRNAs.

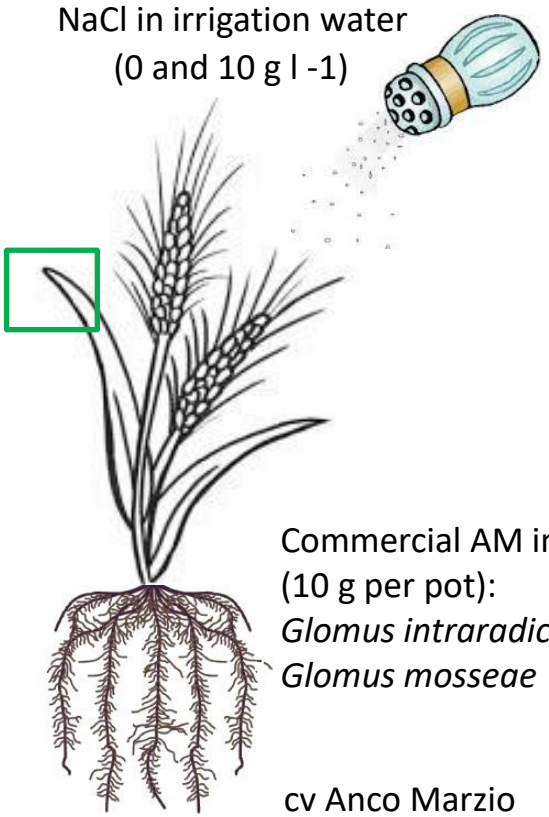
2. This genomic identification of microRNAs together with the analysis of their expression profiles is a well-accepted starting point leading to a better comprehension of the role of *MIR* genes in the genus *Triticum*.

CONTRIBUTION 6

WHEAT MOLECULAR RESPONSES TO SALINITY AND MYCORRHIZAL INOCULATIONS

Aim of this study

to determine how some key genes of durum wheat involved in drought stress responses and nutrient transport are regulated by AMF inoculations in high salinity environment



4 conditions:

- Absence and presence of salinity stress
- With or without AM fungi inoculation

(**No-stress +AM**, **No-stress –AM**, **Saline-stress +AM**, **Saline-stress –AM**)

11 genes analyzed in leaf involved into N transporters and drought stress responses

Trait		No-stress		Saline-stress		Significance		
		+AM	–AM	+AM	–AM	Stress	Inoc.	Stress × Inoc.
No. stems per plant	n°	4.6	5.0	2.7	2.4	***	ns	ns
Aboveground biomass (AB)	g per pot	2.19	2.15	1.51	1.39	***	*	ns
Root biomass	g per pot	2.33	2.14	0.93	0.70	***	*	ns
Proportion of green leaves	% on AB	50.1	46.2	34.3	34.1	***	ns	ns
SPAD value	—	50.5	49.3	53.1	52.1	***	*	ns
MSI	—	86.1	86.0	75.0	66.6	***	ns	*
Mycorrhizal infection	%	36.4	0.8	31.2	0.5	*	***	ns

increase

decrease

Hypothesis

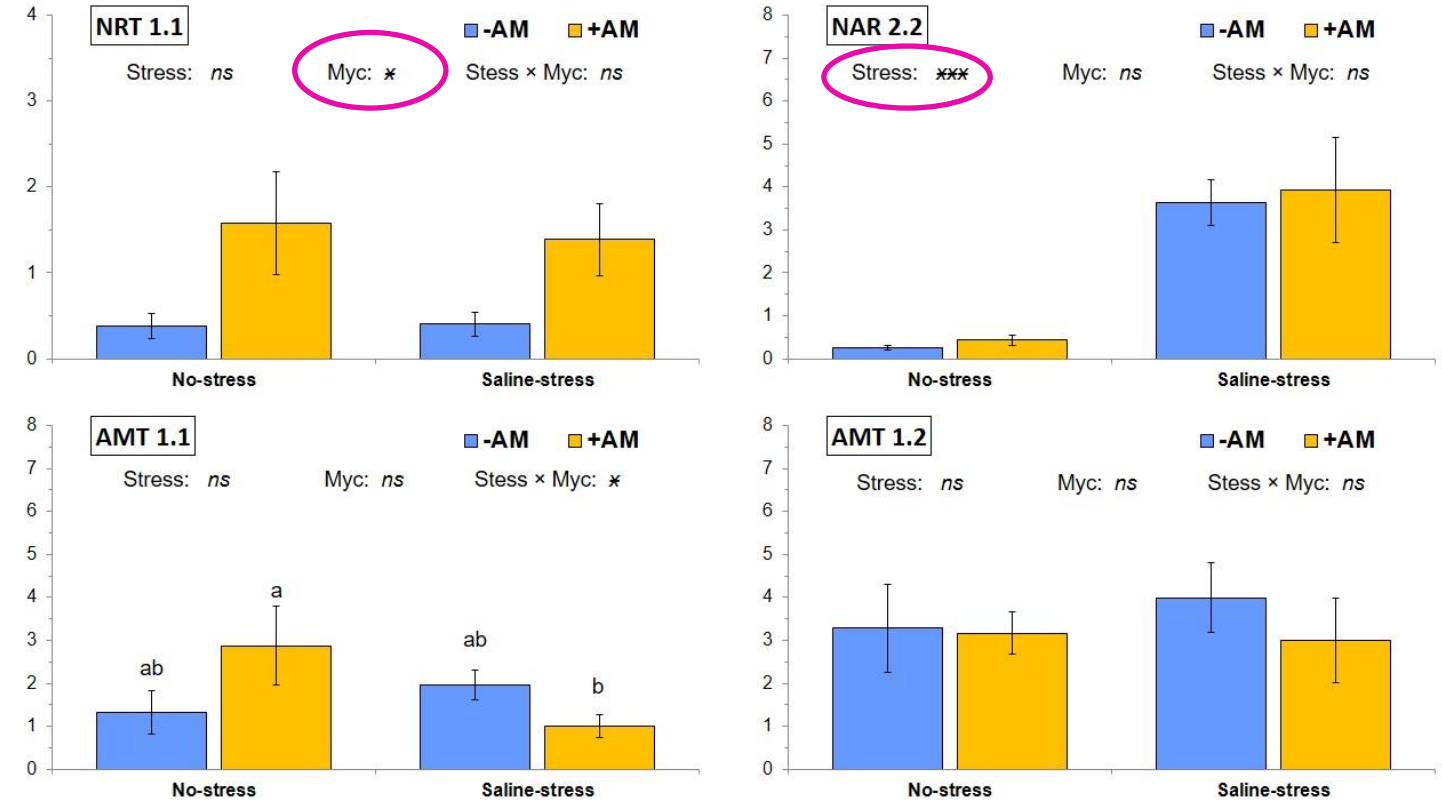
AMF-upregulation of NRT1.1 gene involved into the import of N in leaf tissues from the xylem

Trait	N concentration	No-stress		Saline-stress		Significance		
		+AM	–AM	+AM	–AM	Stress	Inoc.	Stress × Inoc.
N concentration of:								
Total aboveground biomass	g kg^{-1}	0.309	0.265	0.365	0.331	***	***	*
Green leaves	g kg^{-1}	0.385	0.343	0.409	0.389	***	***	*
Senescent and dry leaves	g kg^{-1}	0.204	0.209	0.338	0.359	***	ns	*
Stems	g kg^{-1}	0.251	0.212	0.274	0.232	*	***	ns
Root biomass	g kg^{-1}	0.124	0.122	0.143	0.150	**	ns	ns
Total N uptake	mg N per pot	95.1	82.6	68.5	56.3	***	***	ns

****, **, * denote significant differences at 0.001, 0.01, and 0.05 probability levels, respectively; ns indicate differences not significant*

NITROGEN TRANSPORTER GENES

Their analysis in leaves is very important to determine how N is translocated between leaf tissues and how it is assimilated in leaf cells from the xylem



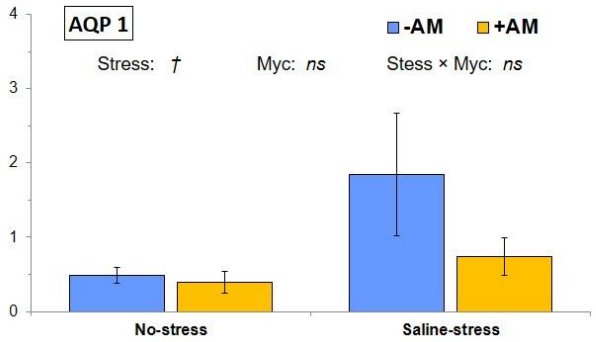
ns = not significant; † = p value 0,1; x = p value 0,05; xx = p value 0,01; xxx = p value 0,001

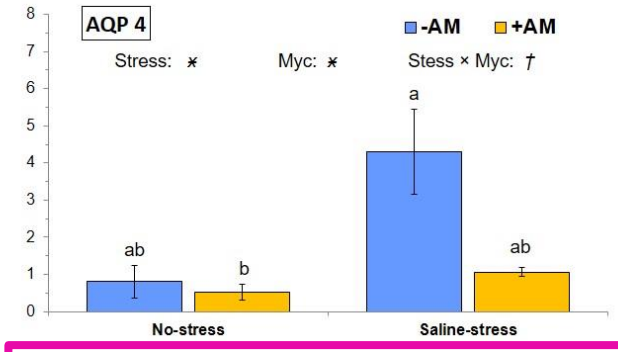
Hypothesis

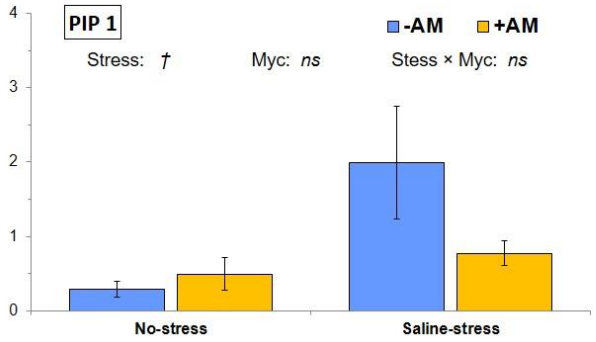
Mycorrhizal inoculation regulate N transport by activating directly N transport genes.

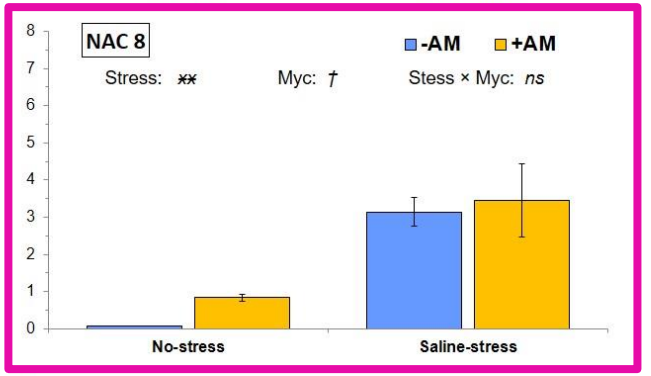
Salinity induces genes involved in the interaction with N transporters

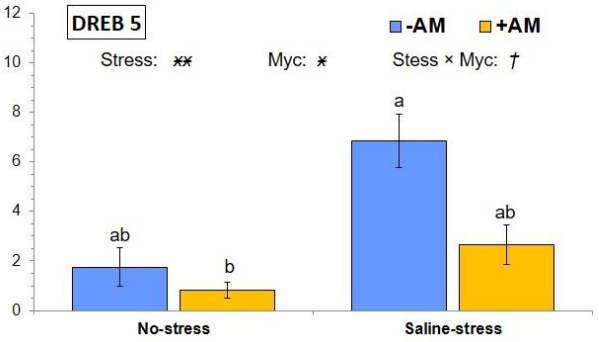
DROUGHT STRESS RESPONSE GENES

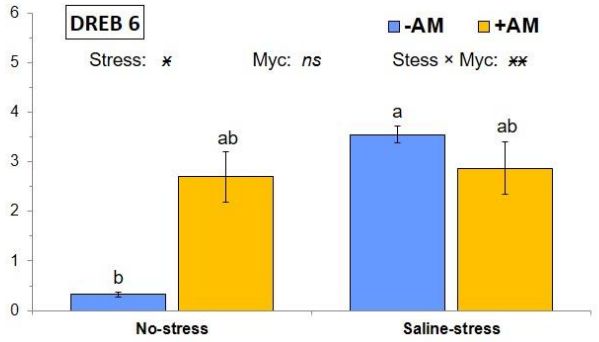


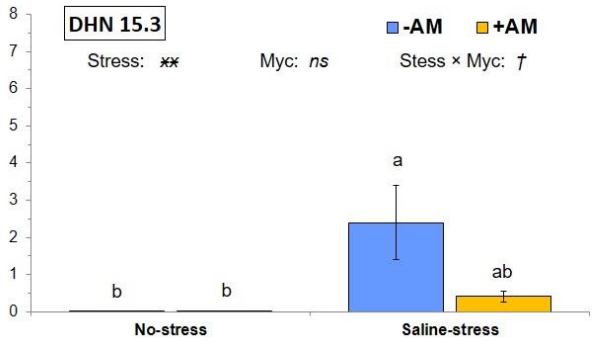












ns = not significant;
† = p value 0,1
⁎ = p value 0,05
⁎⁎ = p value 0,01
⁎⁎⁎ = p value 0,001

Conclusions

- Agronomic benefits from AM symbiosis are confirmed:

Increase of N acquisition and N concentration, aboveground and root biomass, membrane stability → Upregulation of nitrate transporters in leaves such as NRT1.1

- The induction of NAC8 by mycorrhizal inoculation needs further investigation

CONCLUDING REMARKS

1) BIOTIC STRESS

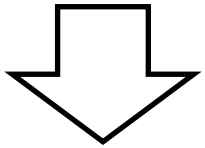
	RPW attacks	Stolbur infection	HLB disease
CARBOHYDRATE METABOLISM			
alpha-amylase		↓	↑
starch synthase		↓	↑
SECONDARY METABOLISM			
Phenylpropanoids genes	↑	↑	
SIGNALING			
DUF 26	↑		↑
LRR III	↓	↓	
HORMONAL PATHWAY			
ethylene	↑	↑	
salicylic acid	↑	↑	↑
ABA pathway	↑	↑	
Allene oxide synthase (JA)	↑	↑	
BIOTIC RESPONSE			
WRKY 40	↑	↑	
WRKY 47	↑	↑	
WRKY 75	↑	↑	

CONCLUDING REMARKS

2) ABIOTIC STRESS

- Durum wheat miRNAome analyzed on Contribution 5 provides a greater global understanding of tissue-specific miRNAs expression in leaf and root tissues

- Data from Contribution 6 confirmed agronomic benefits deriving from AM symbiosis in plants under salt stress condition and showed an induction of some analyzed wheat genes in response to salinity



These are preliminar studies

Further analyses are ongoing to identify miRNAs playing a key role in the well-known benefits of mycorrhizal inoculation in response to drought and salinity stress

Thanks for your attention