Use of molecular markers for quality evaluation in the fresh cut fruit and vegetables distribution chain

Antonio FERRANTE

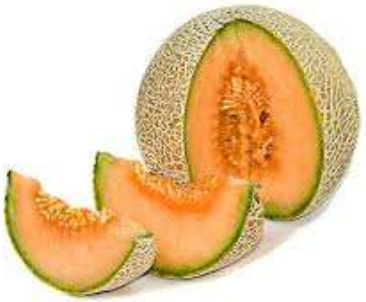
Dept. Agricultural and Environmental Sciences
University of Milano

e-mail: antonio.ferrante@unimi.it

FRESHNESS ⟺ QUALITY

The new challenges of the distribution chains and supermarkets are focused on solving the diatribe between freshness and quality:

- **Freshness** is a defined and quantifiable parameter.
  - It is represented by the period of time from harvest to product preparation;
  - A critical term is to define when the product is not fresh anymore.



- **Quality** is a concept only partially dependent on time because many factors can improve the quality maintenance such as innovation technology.

Quality

Quality is reached in field

Processing can reduce quality

Postharvest quality of horticultural crops

External quality:

- Green colour: **chlorophyll** concentration, carotenoids, but also anthocyanins content.
- Cut surface browning: phenols, polyphenoloxidase (PPO) and peroxidase (PO) enzymes;
- Tissue browning: membrane degradation.

Internal Quality:

-Vitamins: ascorbic acid (Vit. C) and β-carotene (Vit. A);
-Phytonutrients or bioactive compounds: phenols, anthocyanins, carotenoids, glucosinolates, fiber;
-Anti-nutritional or dangerous: nitrates and nitrites.

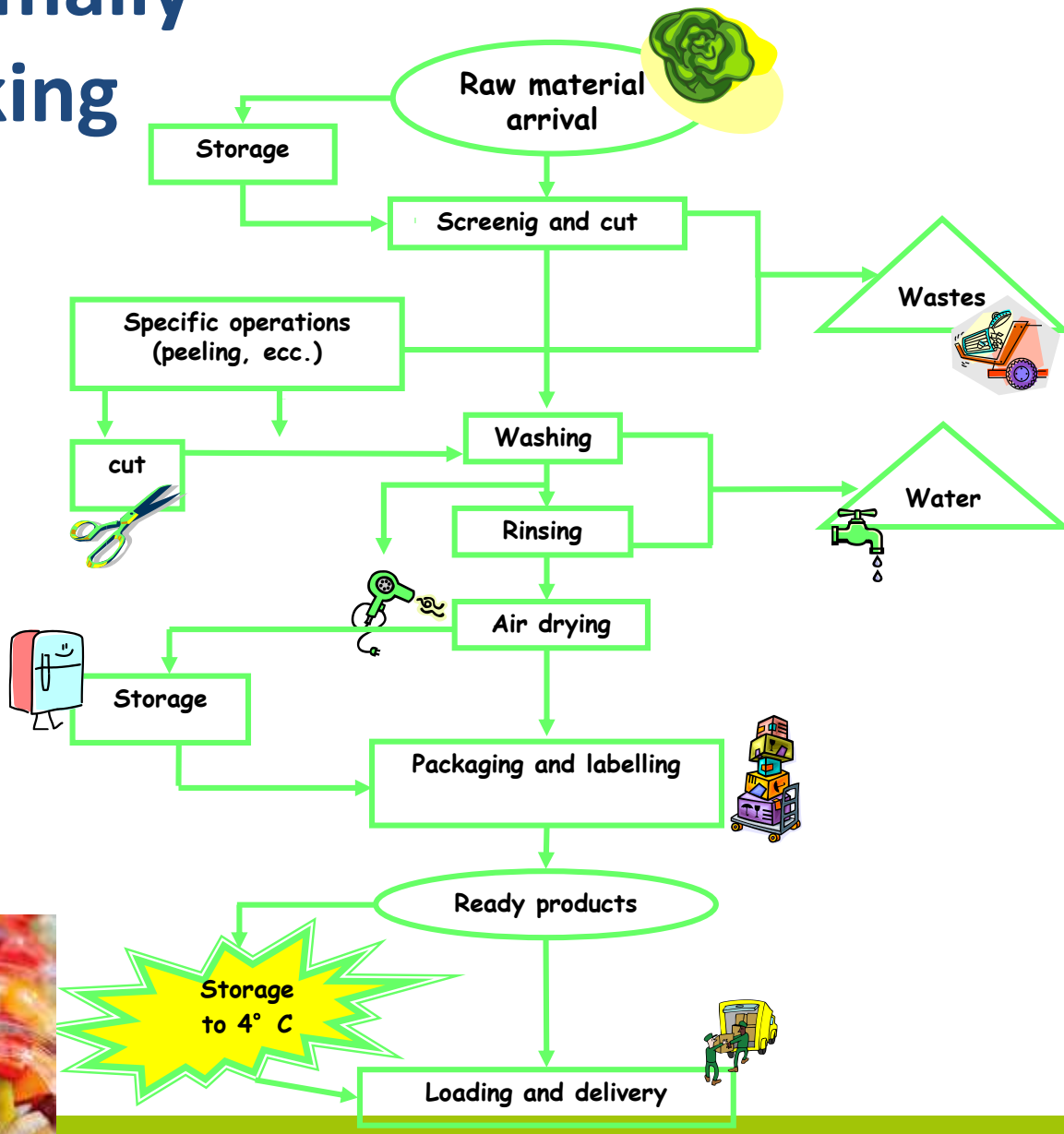
Sanitary quality:

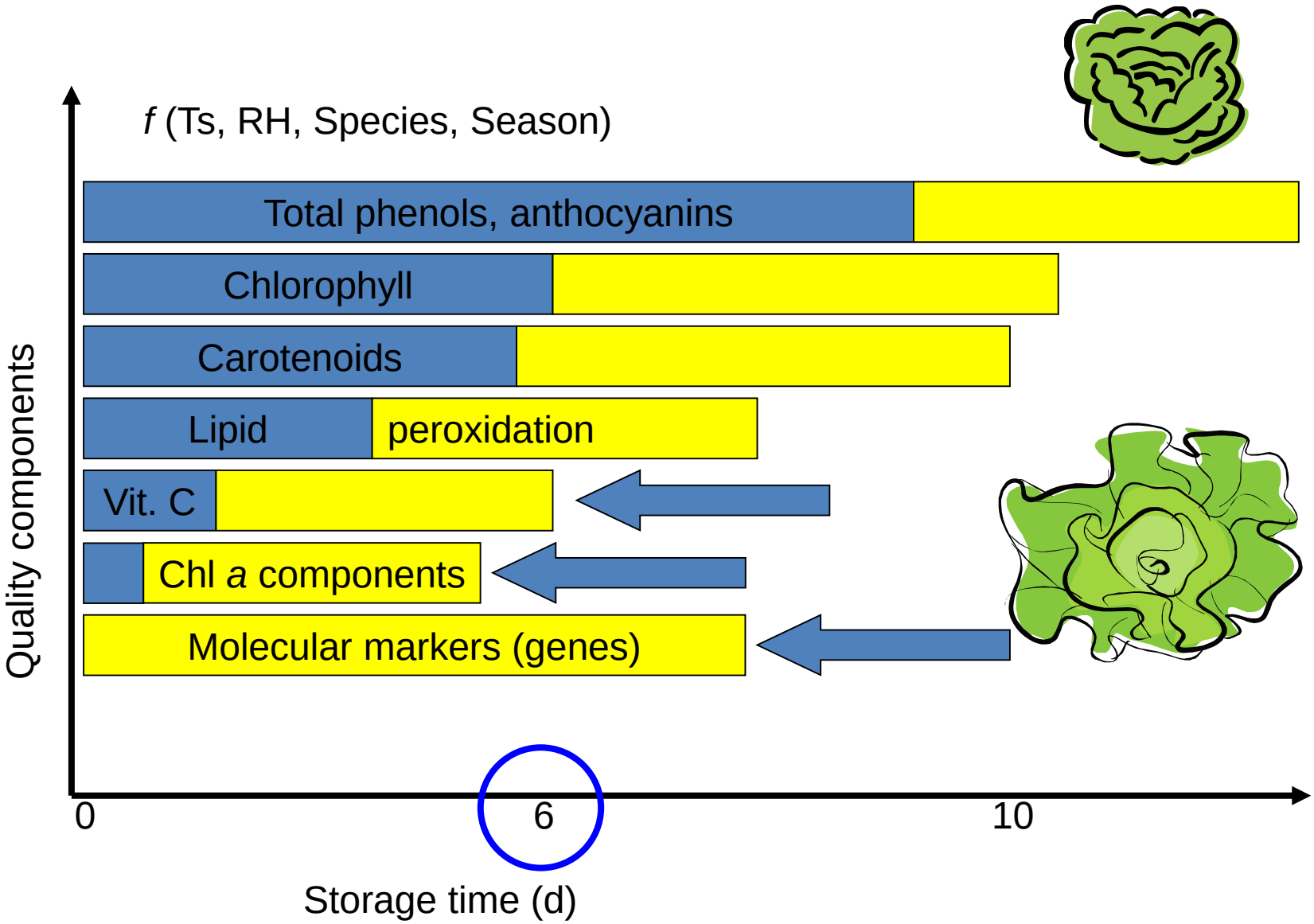
-Pathogens: *E. coli, Lysteria, Salmonella* etc.
-Pesticide residuals
-Pieces of metals, manure etc.

Fresh-Cut or minimally processed working chain

Fruits and vegetables, cut, washed, packed and ready-to-eat :

- about 1/3 *baby leaf*;
- about 2/3 fresh vegetables.




f (Ts, RH, Species, Season)
Total phenols, anthocyanins
Chlorophyll
Carotenoids
Lipid peroxidation
Vit. C
Chl a components
Molecular markers (genes)
Quality components
0
6
10
Storage time (d)

Identification of the best molecular markers

Good quality markers should be specific, reliable and give a rapid answer:

- **Non-destructive**: NIR spectroscopy, chlorophyll *a* fluorescence, mathematical models;

- **Destructive**: monitoring a gene, enzyme, protein or substrate (specific compound).

Objectives of UMIL in QUAFETY

Identification of molecular markers:
-to identify microbial contaminants;
-to evaluate quality.

The research activity is carried out on:
- Rocket (*Diplotaxis tenuifolia* L.);
- Melon (*Cucumis melo* L.).

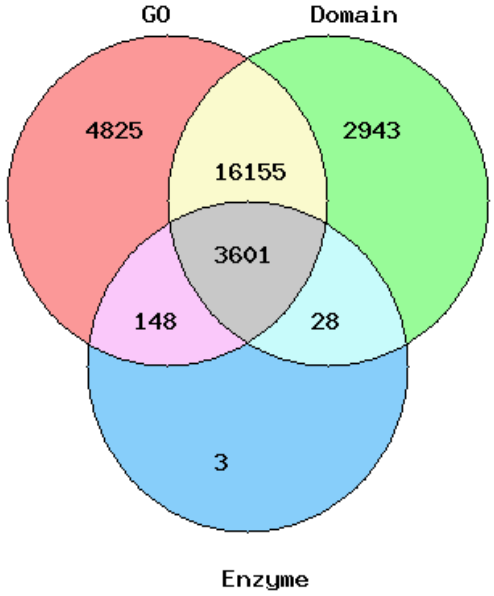
Plant cultivation and stress treatments

Environmental conditions set up:
24-26 ° C, 400 W/m^2 and 16/8 light/night photoperiod. Stresses were imposed for 24 h.

***Pre-harvest stresses*:**
- Salinity 200 mM NaCl;
- Nitrogen deficiency;
- Heat radical stress 40 °C

Post-harvest stresses:
- Dark 20° C
- Water losses 20° C
- Wounding 20° C
- Cold dark storage 4° C

Analysis of the rocket transcriptome (33874 transcripts)

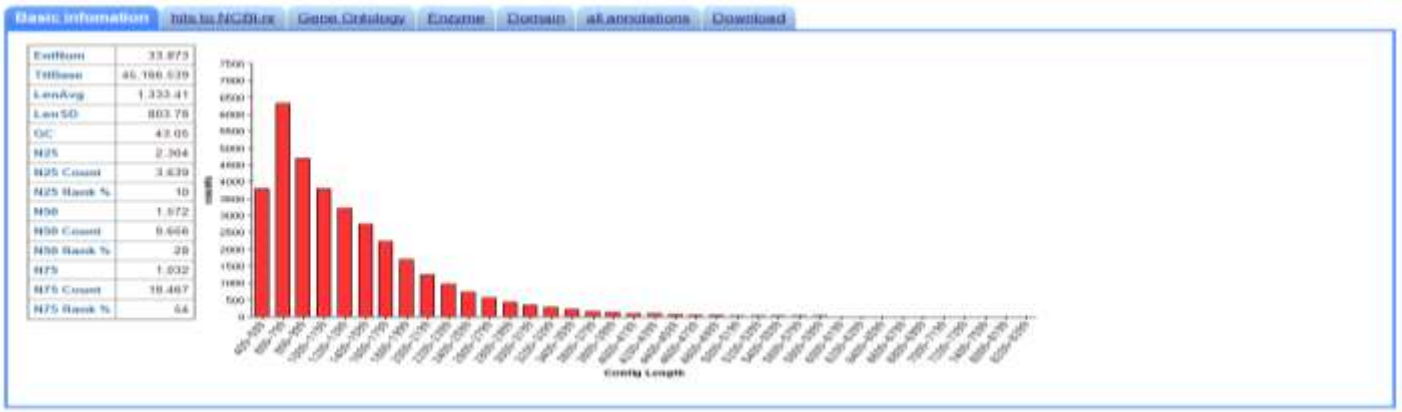


FastAnnotator:

- Basic information (i.e contig lenghts)

Annotation:

- Blast
- Gene ontology
- Enzyme
- Domain



Pre-harvest

Diff. Exp. genes > 2 FC

49 N deficiency

63 Salinity

17 Heat radical stress

vs

Control

vs

Post-harvest

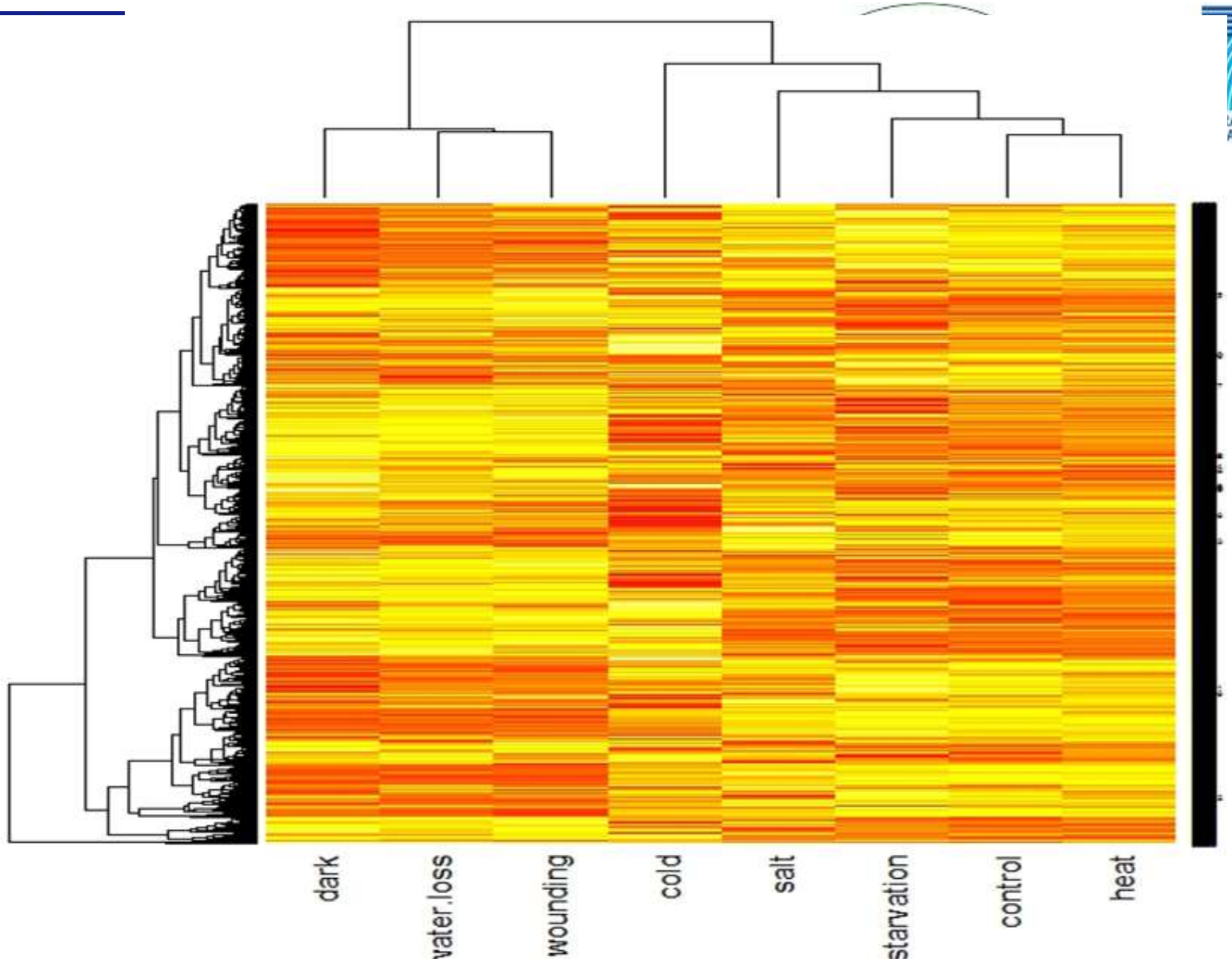
Diff. Exp. genes > 2 FC

Dark **10**

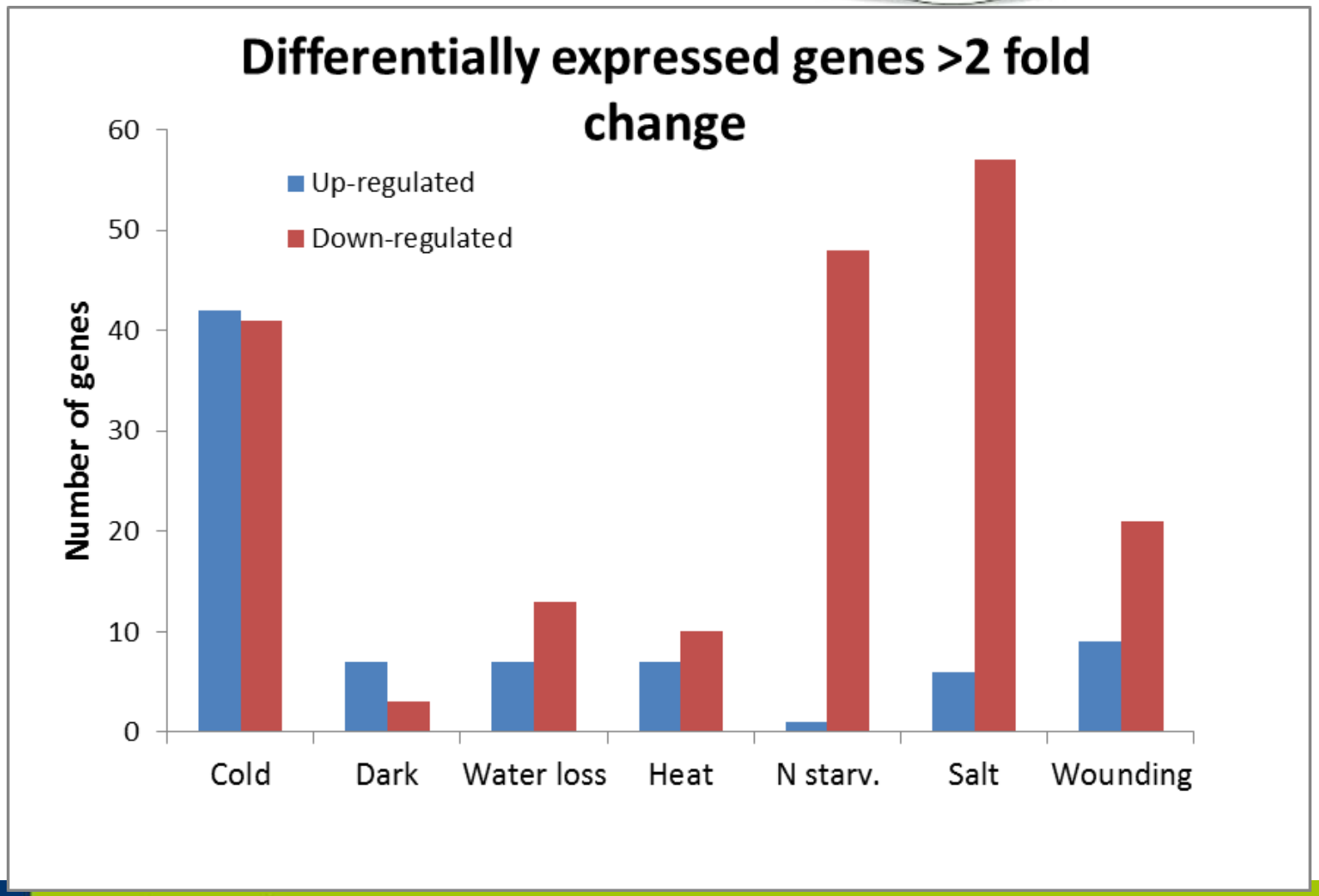
Water loss **20**

Wounding **30**

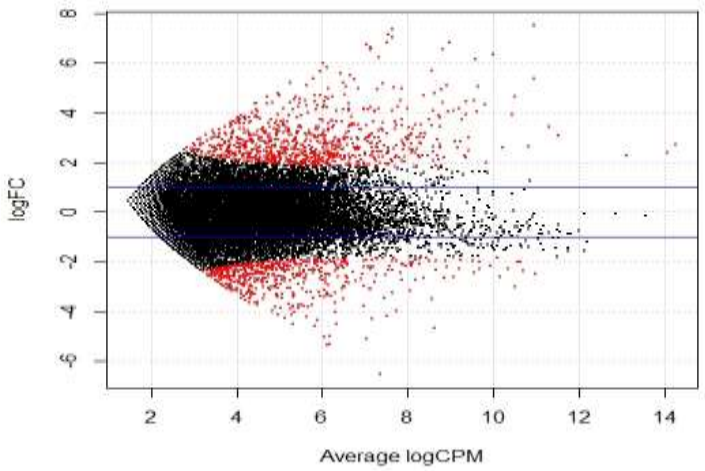
Cold **83**



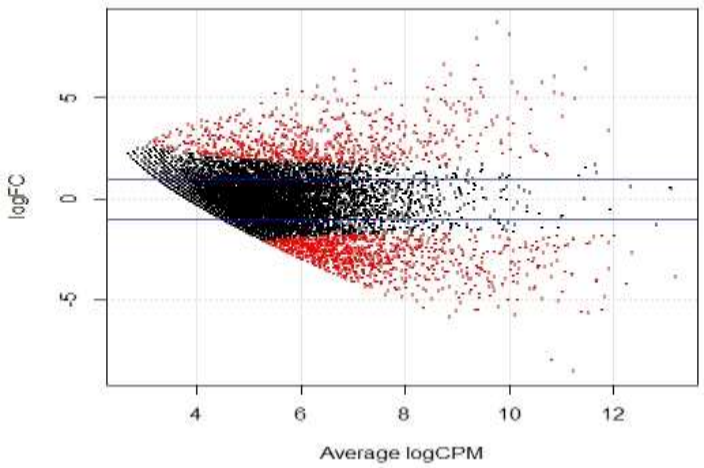
The DEGs between control and stress treatments are shown by heatmap, where dark and light shades indicate higher expression and lower expression respectively.


Differentially expressed genes >2 fold change
Up-regulated
Down-regulated
Number of genes
60
50
40
30
20
10
0
Cold
Dark
Water loss
Heat
N starv.
Salt
Wounding

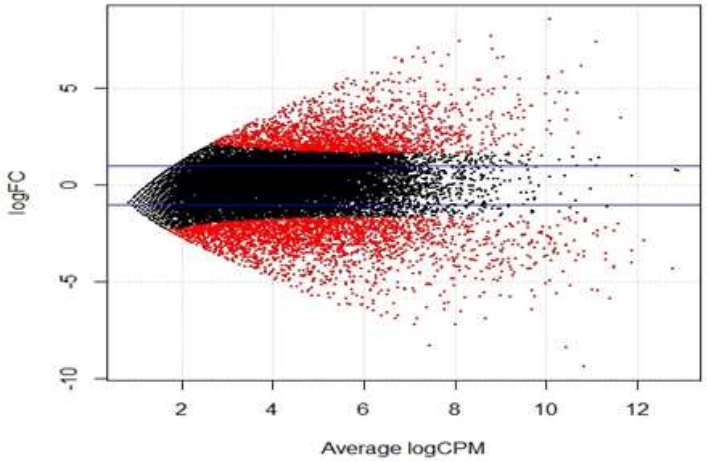
COLD



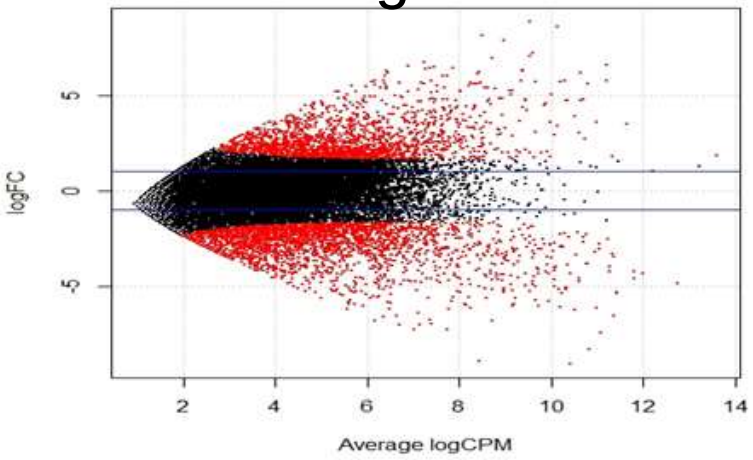
DARK



Water loss



Wounding



Each plot shows the gene expression as log ratio versus abundance of each transcript for a treatment versus the control. Each dot represents a gene and the one marked in red denote DEGs. Blu lines indicate a logFC = 1

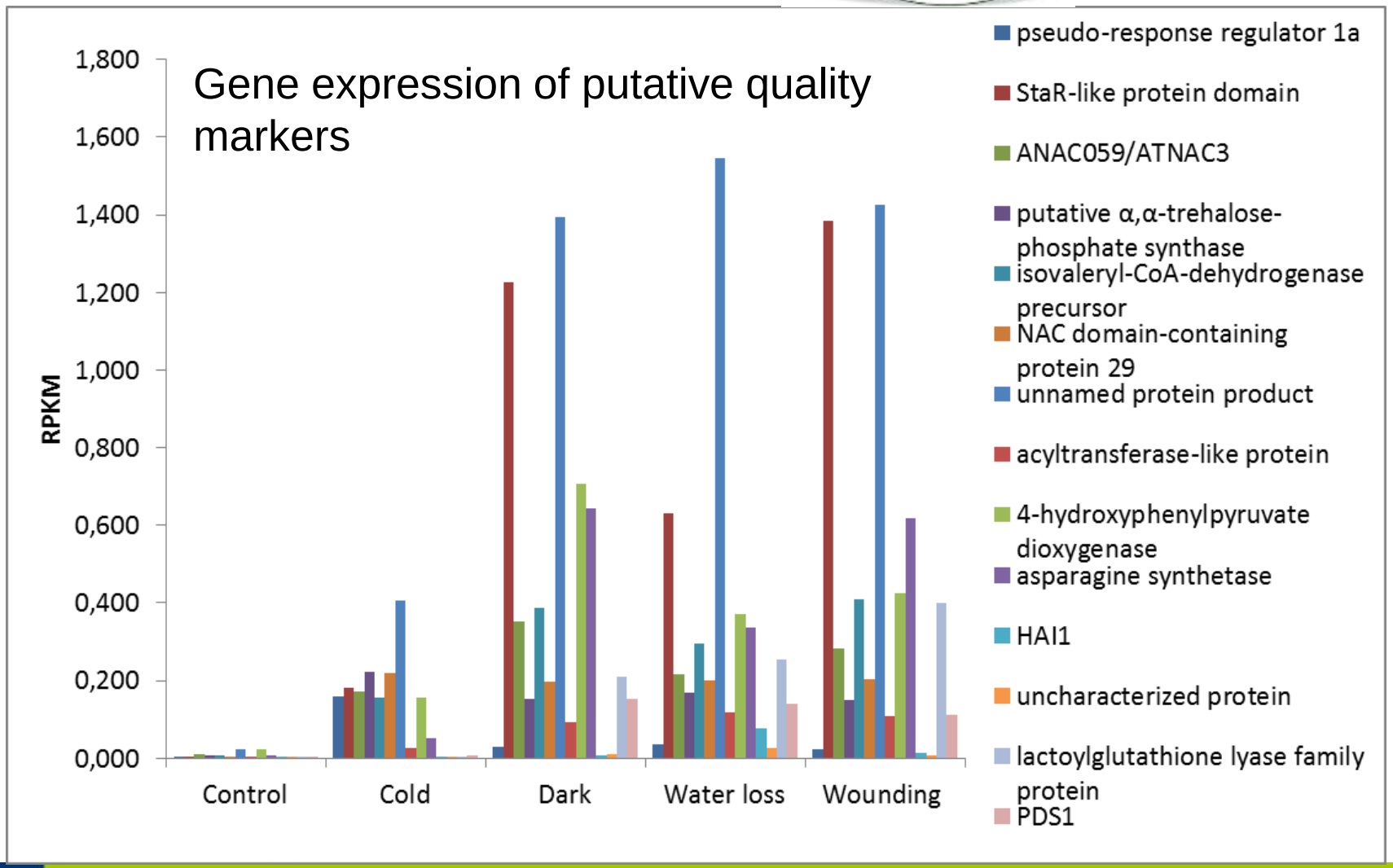
POSTHARVEST STRESSES: GENE EXPRESSION ANALYSIS

Fold-change (log RPKM stress/RPKM control)	Common up-regulated genes	Common stress specific genes (not expressed in the control)	Common down-regulated genes	Common genes that are not expressed in the stresses
> 0	7455	331		
≥ 2.5	378			
≥ 4	69			
≥ 5	19			
≥ 6	13			
≤ -2				
≤ 0			6436	101
≤ -2.5			147	
≤ -4			9	
≤ -5			4	
≤ -6			1	

Isolation of quality markers

The best markers should have the following features:

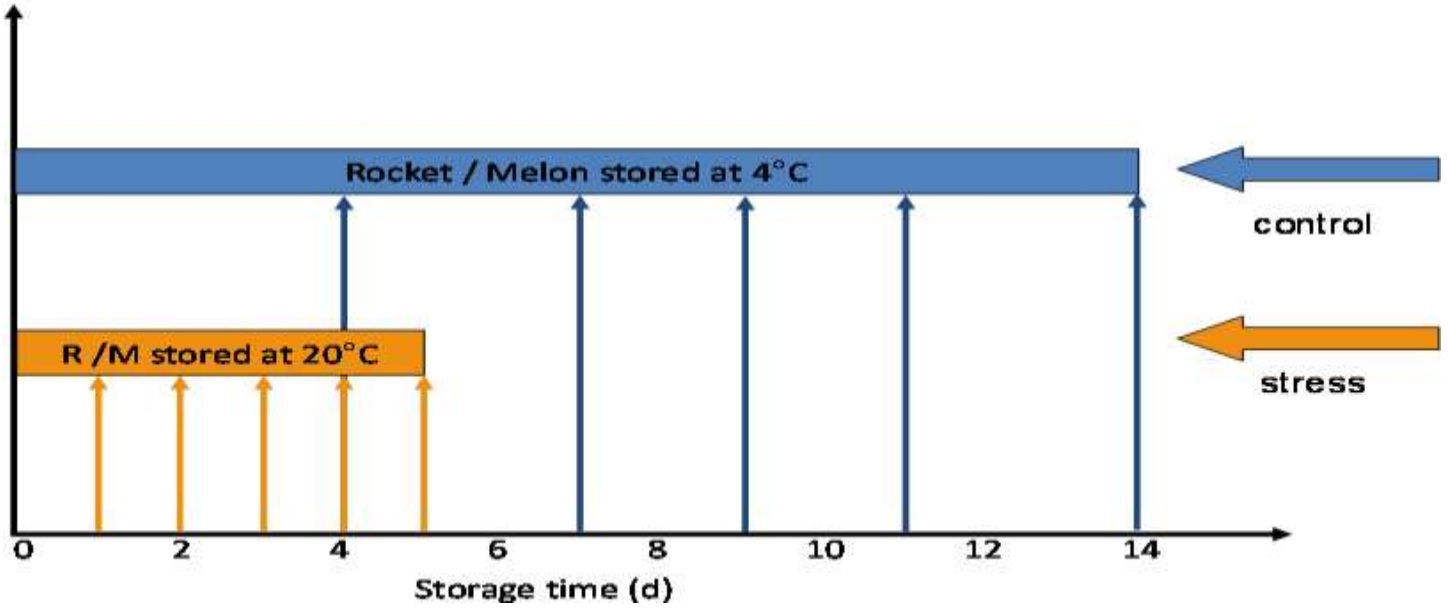
- up-regulation in all stresses responsible to quality losses
- the over-expression must be consistent, fold change >2 or even higher (>4);
- identification of potential markers that have homologs in melon;
- study the structure of putative protein;
- confirm the expression in all stressed samples by qRT-PCR;
- design antibody arrays.

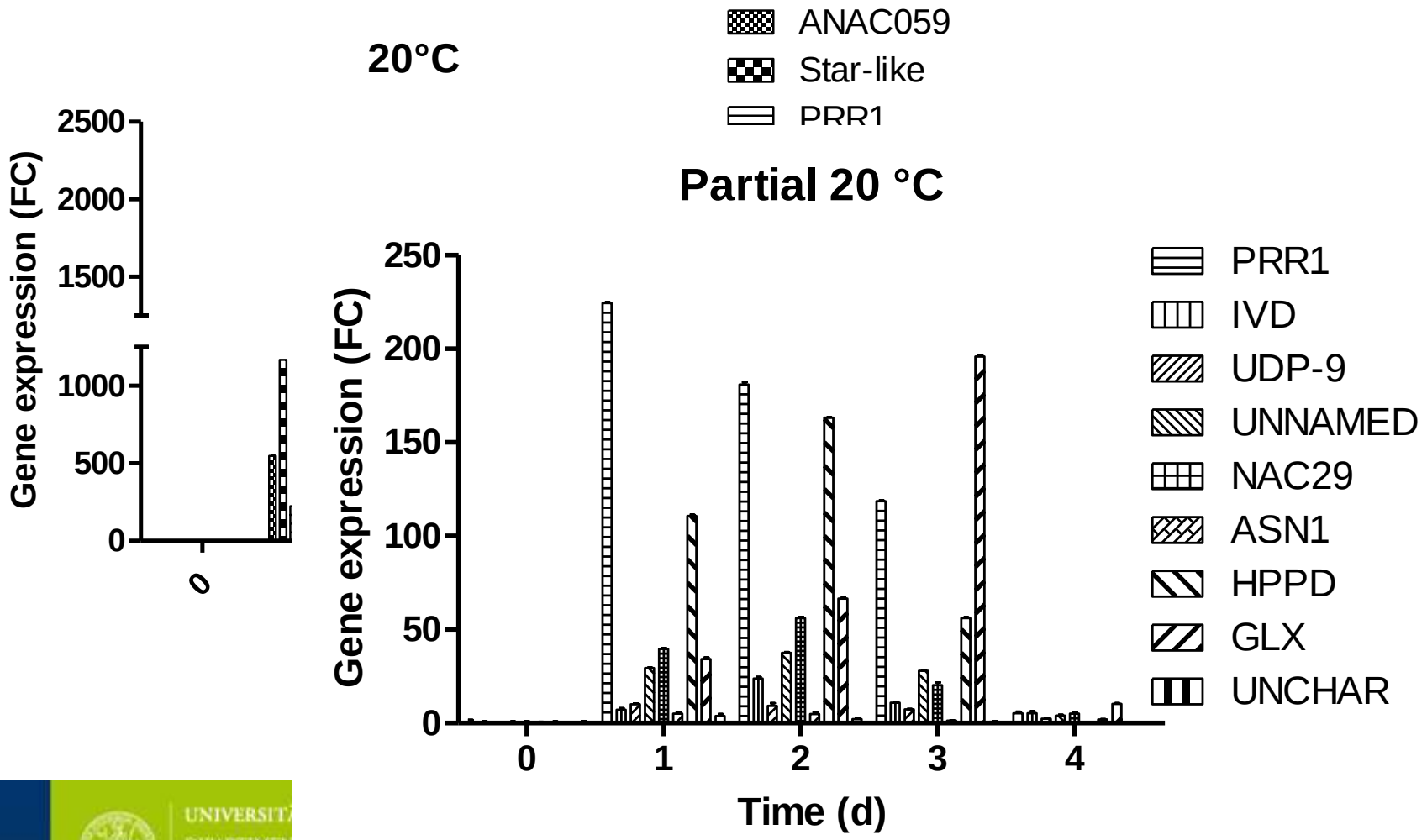

Gene expression of putative quality markers
RPKM
1,800
1,600
1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
Control
Cold
Dark
Water loss
Wounding
pseudo-response regulator 1a
StaR-like protein domain
ANAC059/ATNAC3
putative α,α-trehalose-phosphate synthase
isovaleryl-CoA-dehydrogenase precursor
NAC domain-containing protein 29
unnamed protein product
acyltransferase-like protein
4-hydroxyphenylpyruvate dioxygenase
asparagine synthetase
HAI1
uncharacterized protein
lactoylglutathione lyase family protein
PDS1

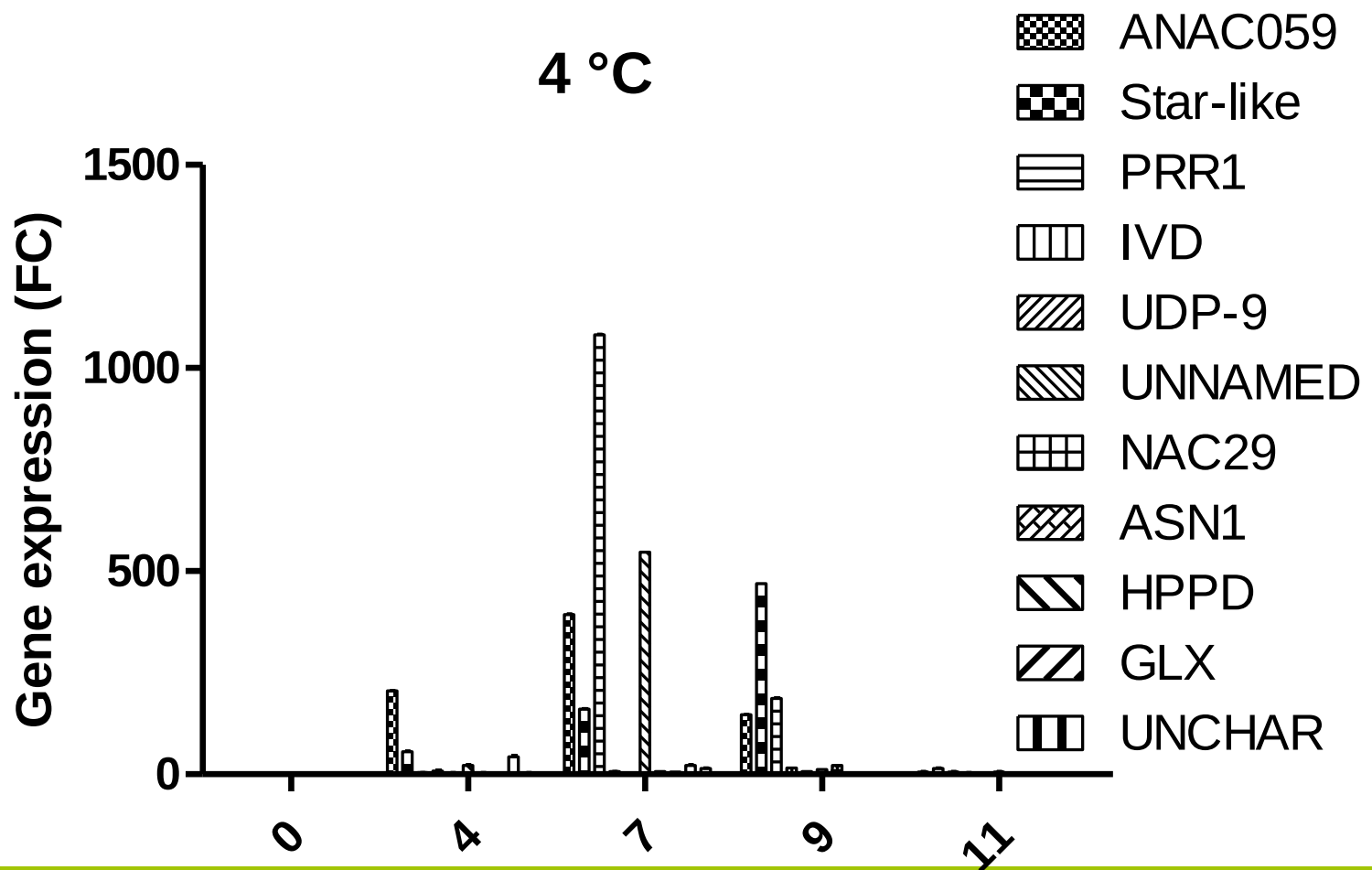
Determination of quality parameters in post-harvest

Rocket: *Diplotaxis tenuifolia* L.
Melon: (*Cucumis melo* L.)
Cv. Macigno
Cv. Pregiato

Sampling time




ANAC059
Star-like
PRR1
20°C
Gene expression (FC)
2500
2000
1500
1000
500
0
0
Partial 20 °C
Gene expression (FC)
250
200
150
100
50
0
0
1
2
3
4
Time (d)
PRR1
IVD
UDP-9
UNNAMED
NAC29
ASN1
HPPD
GLX
UNCHAR


4 °C
Gene expression (FC)
1500
1000
500
0
0
4
7
9
11
Time (d)
ANAC059
Star-like
PRR1
IVD
UDP-9
UNNAMED
NAC29
ASN1
HPPD
GLX
UNCHAR

Determination quality parameters

The potential markers will be analyzed and correlated with physiological determinations:

- Ascorbic acid content (HPLC determination);
- Abscisic acid content (ELISA)
- Chlorophyll, carotenoids content (spectrophotometric);
- Chlorophyll *a* fluorescence (Portable fluorimeter);
- Ethylene measurements (GS);
- Lipid peroxidation (membrane integrity - spectrophotometric).

Handy PEA (Plant Efficiency Analyser)

High resolution instrument (10 µs) and high amount of data recording capacity.

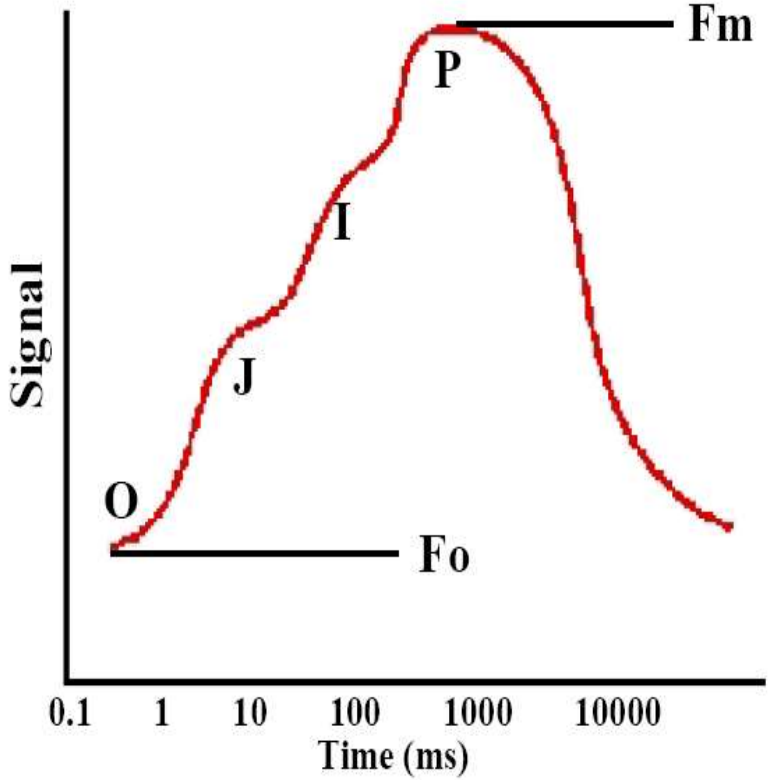
Samples (leaves) were dark adapted for 10-30 min, to allow the oxidation of plastoquinone electron acceptor pool (Qa).

Samples were exposed to flash of saturing light 3000 µmol m^{-2} s^{-1}, using LED with maximum emission peak at 650 nm.

Detector: Fast response PIN photodiode con RG9 long pass filter.

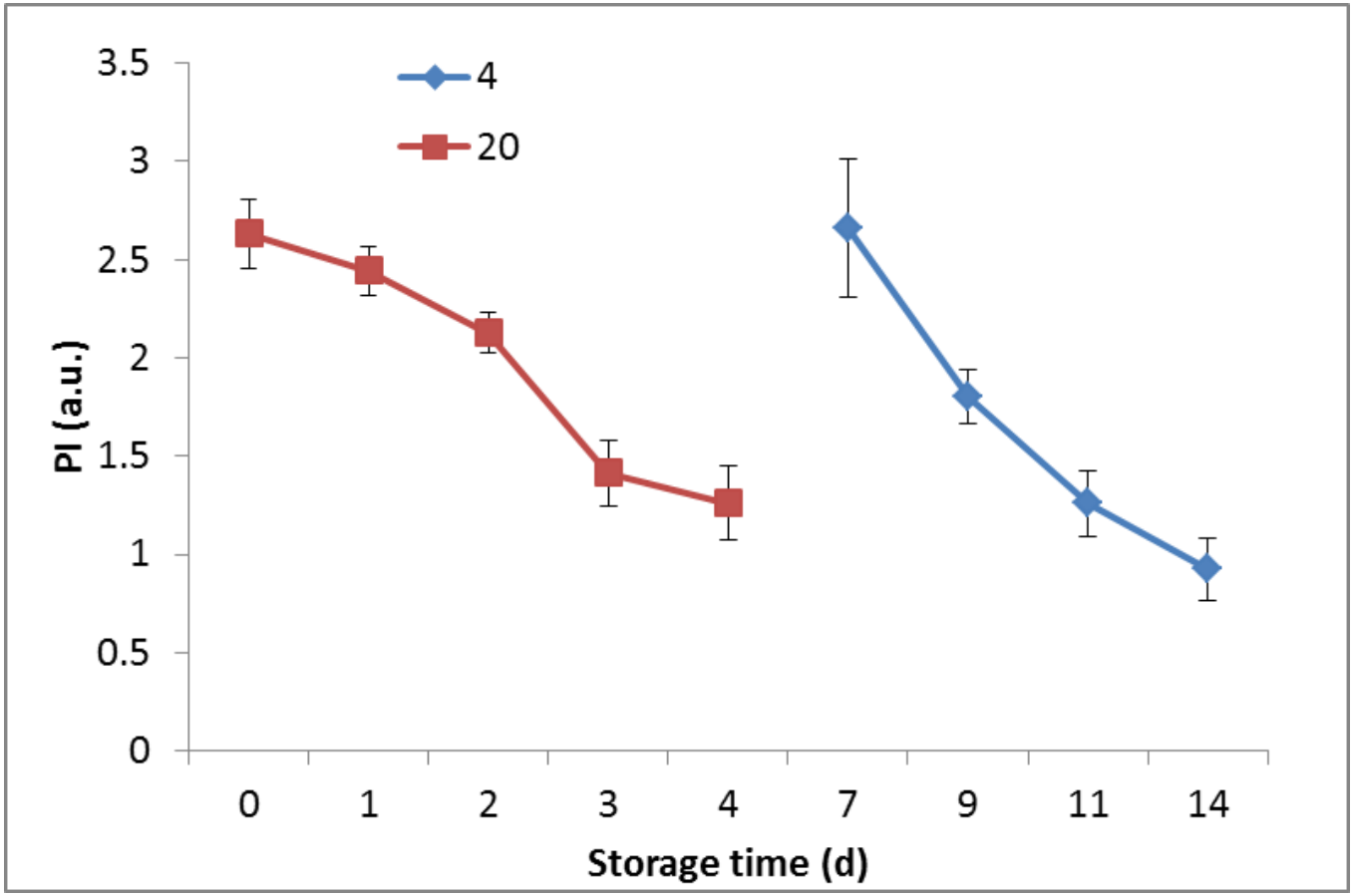
Measured and calculated parameters

- **Fo** – fluorescence level when plastoquinone electron acceptor pool (Qa) is fully oxidised.
- **Fm** – fluorescence level when Qa is transiently fully reduced.
- **Fv** – variable fluorescence (Fm-Fo).
- **Fv/Fm** – maximum quantum efficiency of photosystem II (<0.83).
- **Tfm** – time at which Fm occurs.

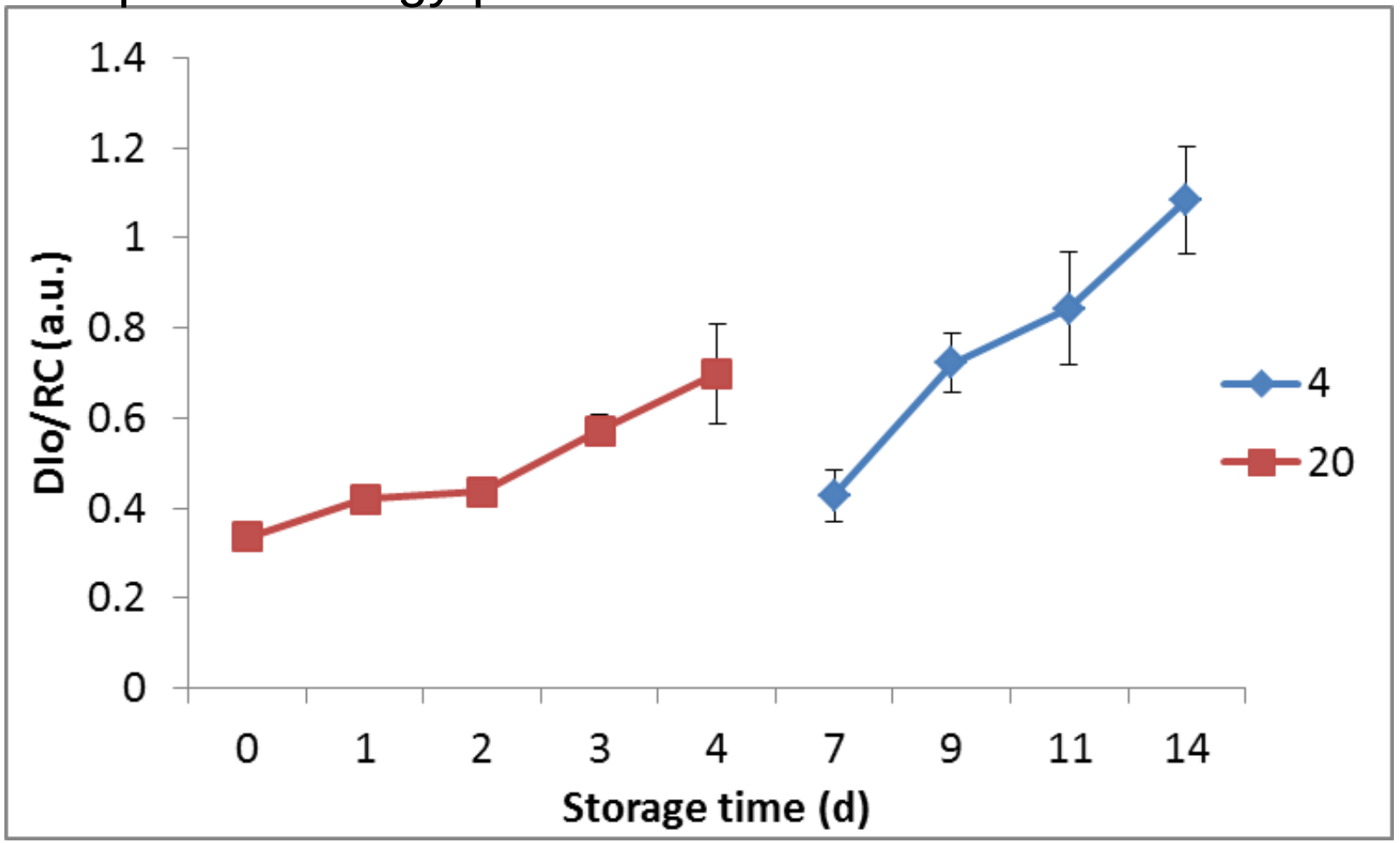


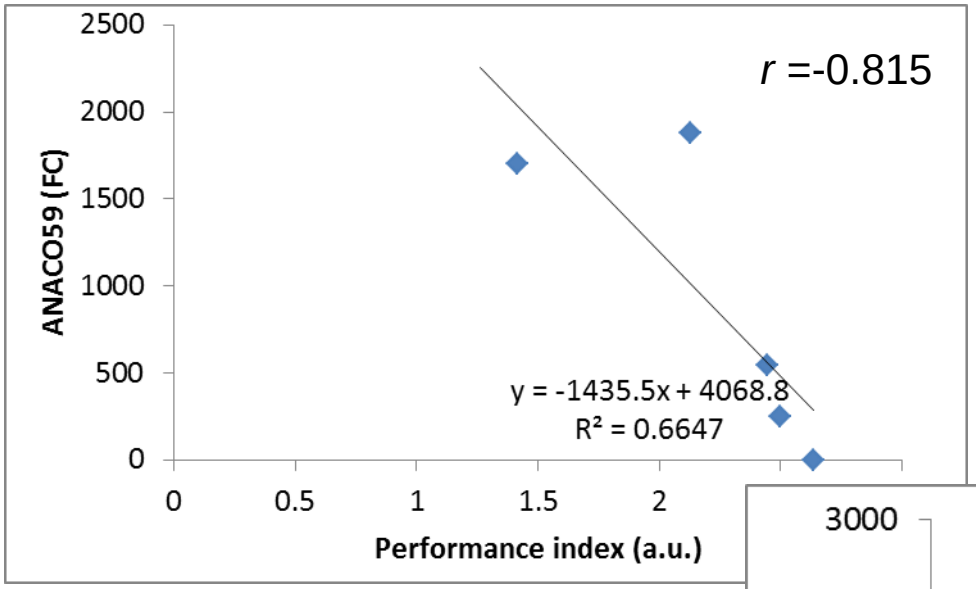
OJIP Fluorescence Induction Curve

Performance index (PI) quantifies the main steps in photosystem II (PSII) photochemistry including light energy absorption, excitation energy trapping, and conversion of excitation energy into electron flow.

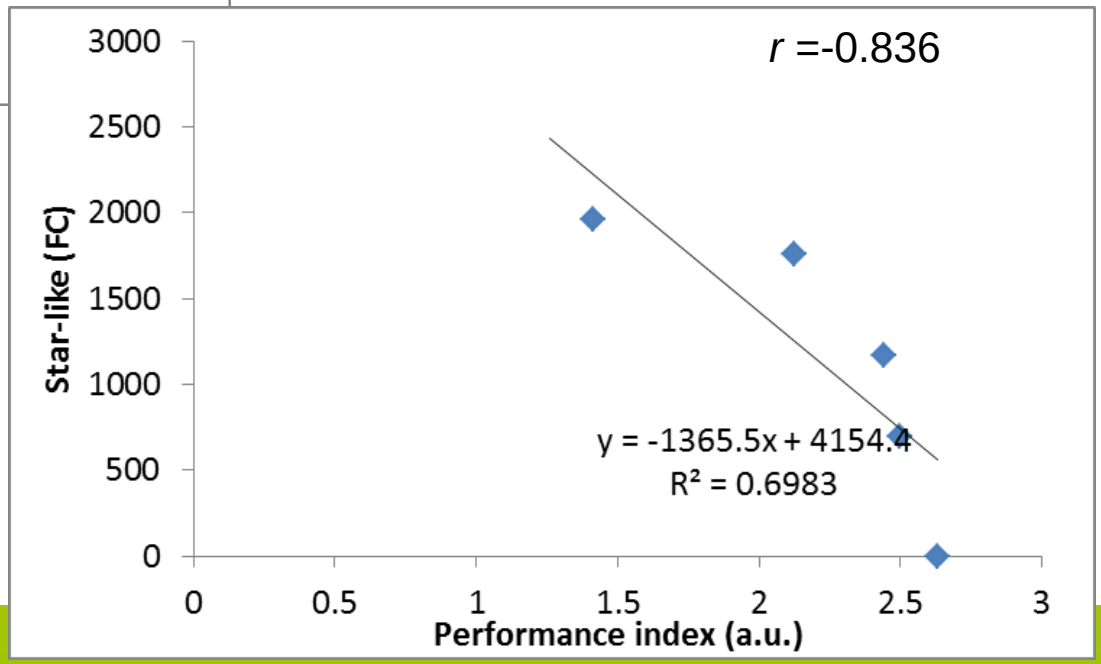


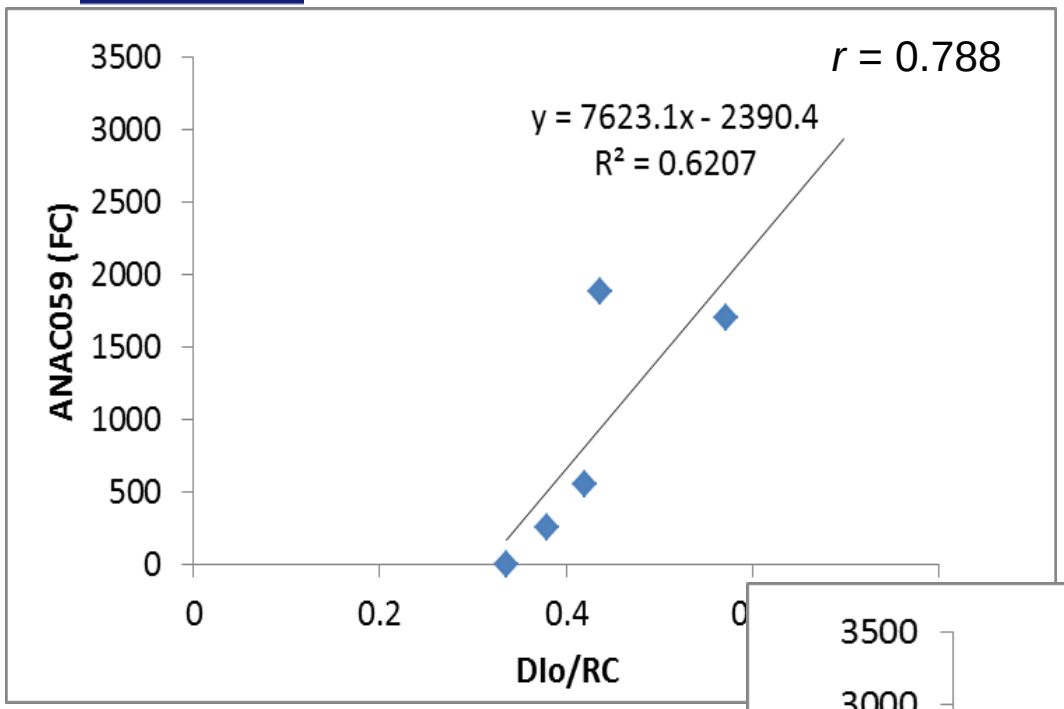
Dissipation energy per reaction centre



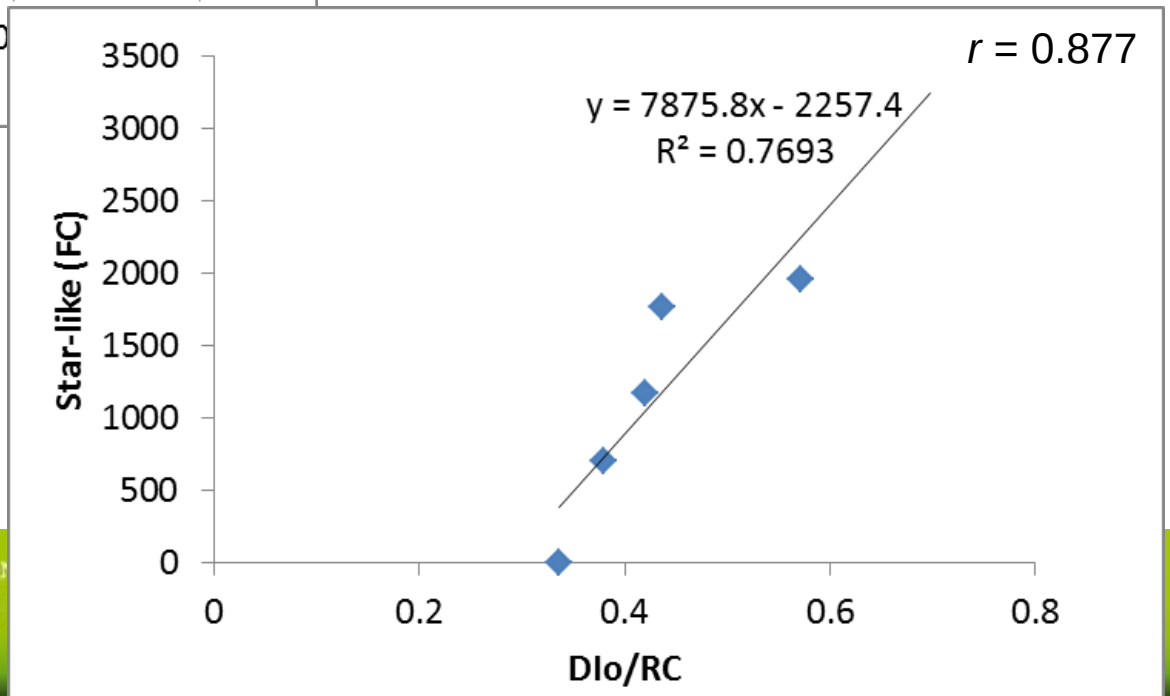


Correlation analysis between putative quality marker and chlorophyll *a* fluorescence derived index.





Correlation analysis between putative quality marker and chlorophyll *a* fluorescence derived index.



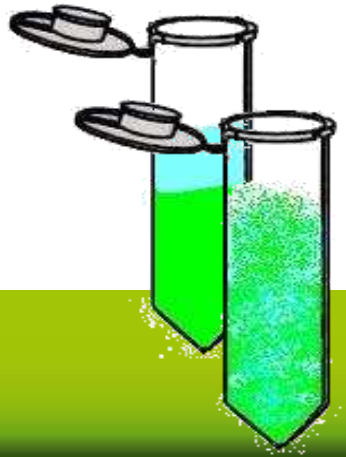
UNIVERSITÀ DEGLI STUDI DI MILA
DIPARTIMENTO DI SCIENZE AGRARIE E AMBIENTALI - PRODUZIONE, TERRITORIO, AGROENERGIA

Correlation matrix among quality physiological parameters and molecular markers in rocket

	DtNAC3	*DtStAR*	*DtPRR*	*DtIVD*	*DtTPS*	*DtUPP*	*DtNAC29*	*DtASN*	*DtHPD*	*DtGLX*	*DtUPF*
Fv/Fm	-0.0264	-0.6245 ****	-0.4948 ****	0.1194	0.3415 ***	0.06562	0.3461 ***	0.1205	0.1522	0.1685	0.4151 ****
PI	0.0121	-0.5145 ****	-0.3239 **	0.0114	0.2463 *	0.1239	0.2917 **	-0.1098	0.08425	-0.0456	0.5160 ****
DLo/RC	0.0117	0.5831 **	0.4749	-0.1354	-0.3402	-0.0658	-0.3360	-0.1347	-0.1636	-0.1777	-0.3761 ***
DLo/CS	0.0974	0.6393 ****	0.5866 ****	-0.1320	-0.3275 **	0.0959	-0.3163 **	-0.1931	-0.1508	-0.2194 *	-0.2777 **
RC/CSo	0.1544	-0.1915	-0.0887	0.0318	0.1548	0.2025	0.1879	-0.0781	0.1103	-0.0317	0.3305 **
RC/CSm	0.0298	-0.5533 ****	-0.4114 ****	0.0357	0.26 *	0.0889	0.3136 **	-0.0213	0.0974	0.0395	0.4737 ****
TBARS	0.0667	-0.1518	-0.4486	0.062	0.0722	-0.2382	0.04429	0.3407	-0.0151	0.2962	-0.2446
TS	-0.1880	-0.7290 *	-0.5640	-0.1802	0.1307	0.1377	0.2084	-0.017	-0.1224	0.030	0.4870
RS	-0.0870	-0.7289 *	-0.6508	-0.006	0.3880	-0.0136	0.4047	0.1956	0.01048	0.2567	0.5779
SUC	0.4656	-0.2520	-0.2040	0.2003	0.6055	0.4826	0.6417	-0.2990	0.2990	-0.1700	0.9053 ***
AsA	0.0603	0.1367	-0.0749	0.4013	0.3367	0.1357	0.4824	0.0654	0.04617	0.1694	0.1994
ET b	0.2212	0.0376 *	-0.1158	0.7281	0.6897	0.6119	0.7441	0.4005	0.2207	0.5331	0.3623
ET a	-0.3045	-0.7819	-0.6557	-0.1234 *	0.1403 *	-0.0792	0.2701 *	0.1382	-0.3297	0.2070	0.4814
H_2O_2	0.1177	0.1187	0.3968	-0.0829	-0.0157	0.2739	-0.2564	0.0442	0.2895	-0.0217	-0.0390
O_2^-	-0.4504	0.4419	0.3319	0.1867	-0.0491	-0.1934	-0.1702	-0.2419	-0.2641	-0.2926	-0.4284

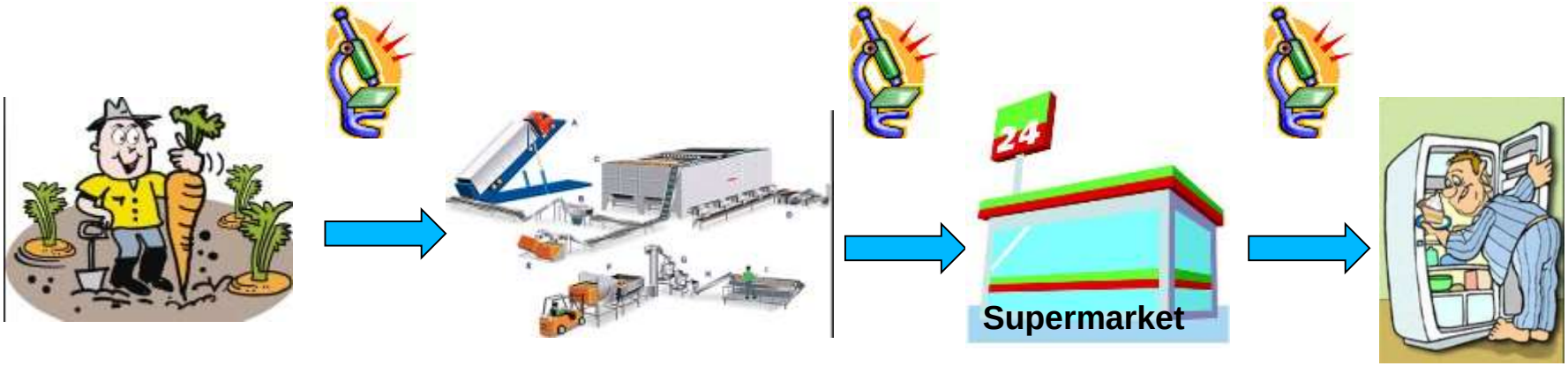
Final scope

Scale down all the complex assay procedures in order to use directly crude protein extracts of fresh cut vegetables for microbial and quality evaluation.



Conclusions

The transcriptome data provide a wide number of possible genes that can be tested as quality markers. In the future genes up-regulated under postharvest stresses can be used to asses shelf life quality, while genes up-regulated under pre-harvest stresses can be a valuable support in the quality evaluation of raw materials.



Marina Cavaiuolo

Giacomo Cocetta

Roberta Bulgari

Anna Spinardi

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE AGRARIE
E AMBIENTALI - PRODUZIONE,
TERRITORIO, AGROENERGIA