CIHEAM - International Centre for Advanced Mediterranean Agronomic Studies

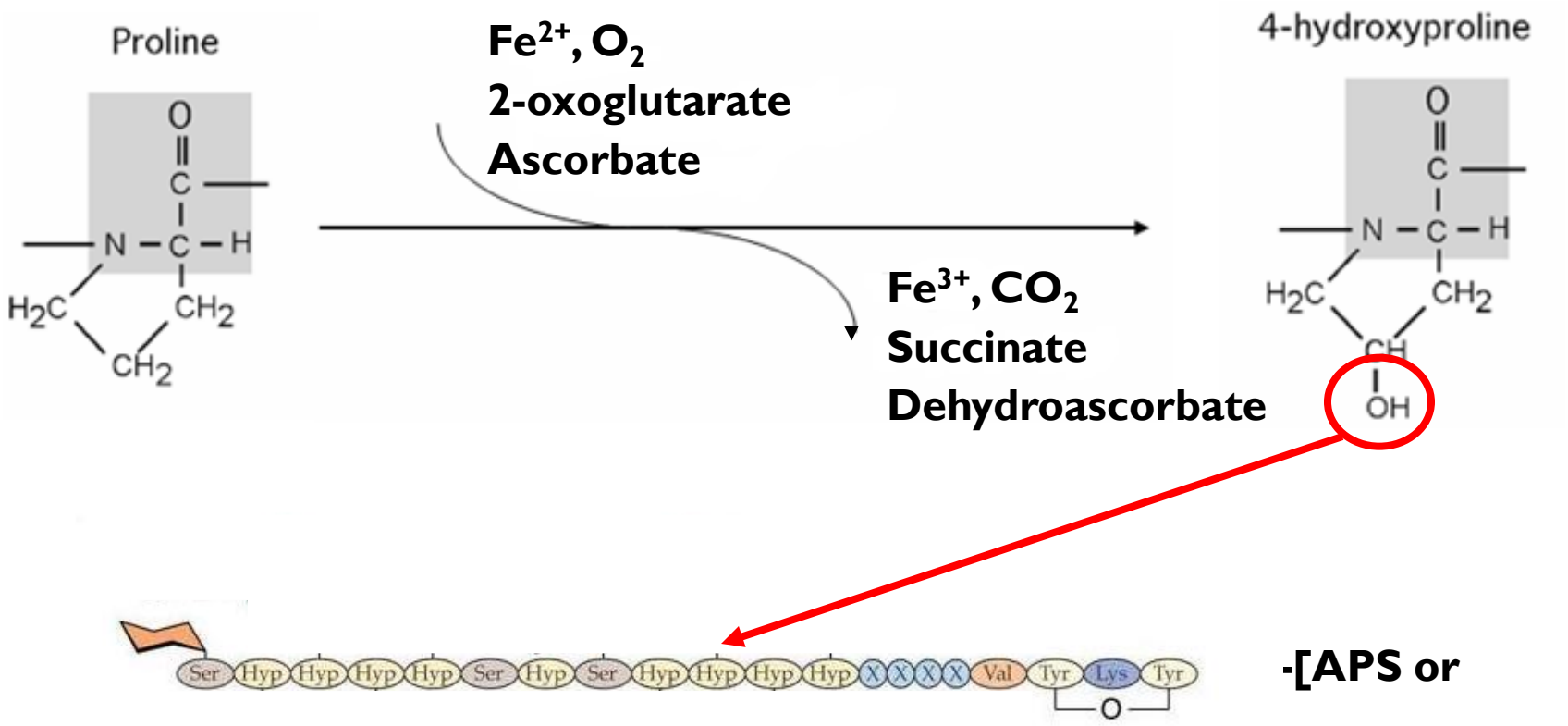
Mediterranean Agronomic Institute of Chania

SUPPRESSION OF A TOMATO PROLYL 4 HYDROXYLASE RESULTS IN MULTIPLE ALTERATIONS ON FRUIT DEVELOPMENT, RIPENING AND HEALTH COMPONENTS

Perrakis A.[1], Craita B.E.[1], El Zein Z.[1], Kaloudas D.[1], Krokida A.[1], Arhondakis S.[1], MOURTZINOS, I.[2] Kalaitzis P.[1]

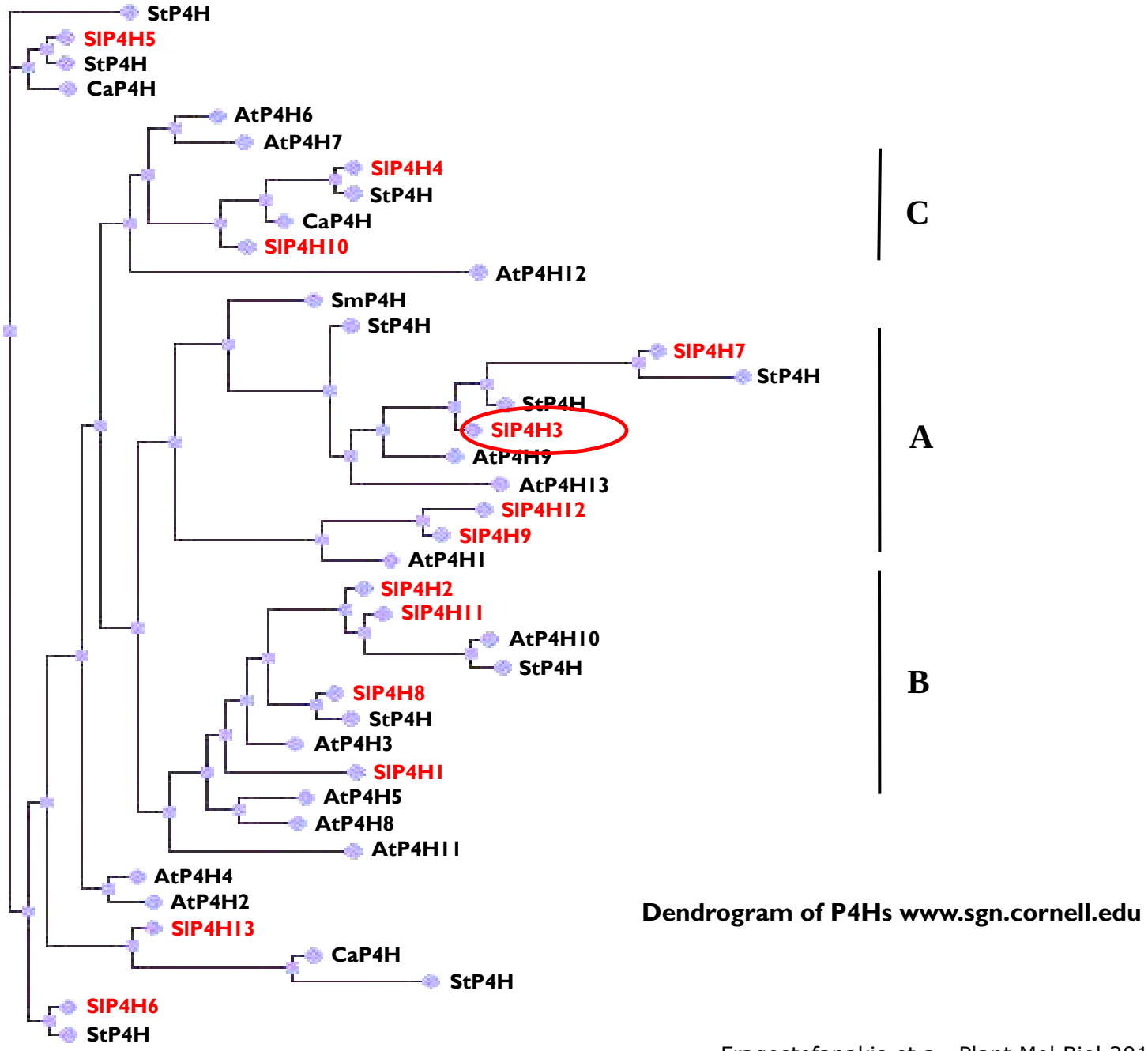
[1]Department of Horticultural Genetics, MAICH, Chania, Greece
2Dept of Food Science, Agronomy School, Aristotle University of Thessaloniki

Enzymatic Activity of Prolyl 4 Hydroxylases



Pr
hy **-[APS or**

[not basic]–[not T]–[AVSG]–Hyp–[AVST]–[GAVPSTC]–[APS]

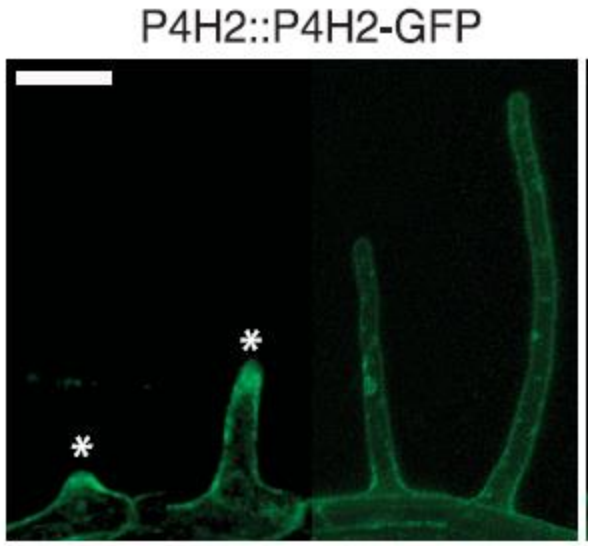


Dendrogram of P4Hs www.sgn.cornell.edu

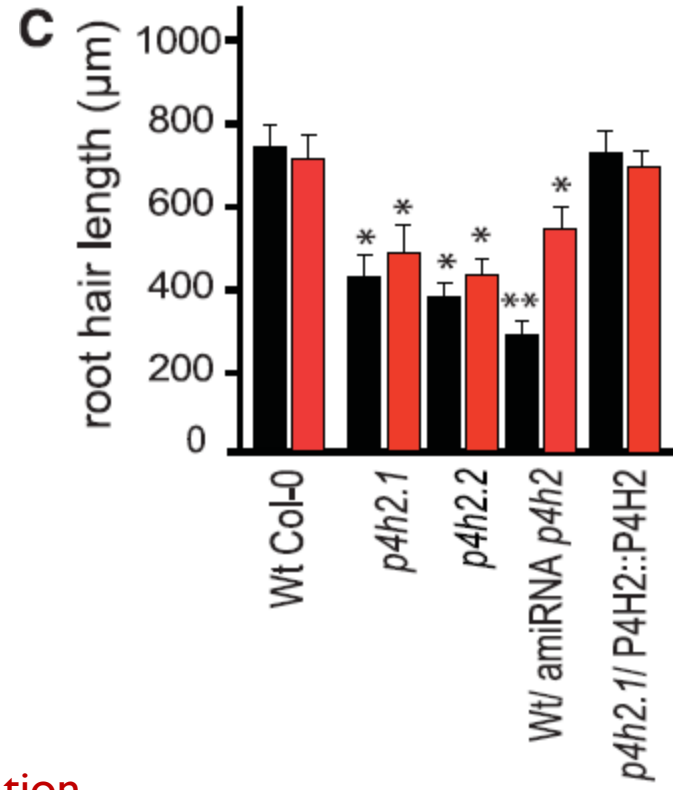
Fragostefanakis et a., Plant Mol Biol 2014

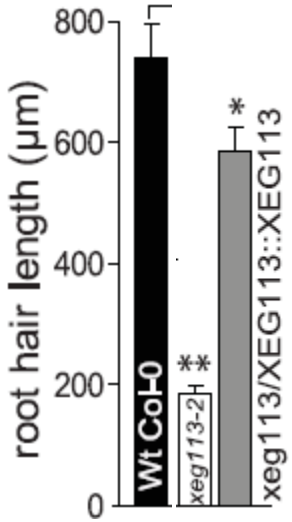
The posttranslational modifications of O-glycosylated cell wall proteins are of broad importance to cell differentiation and plant development

Arabidopsis P4Hs



Root hair development



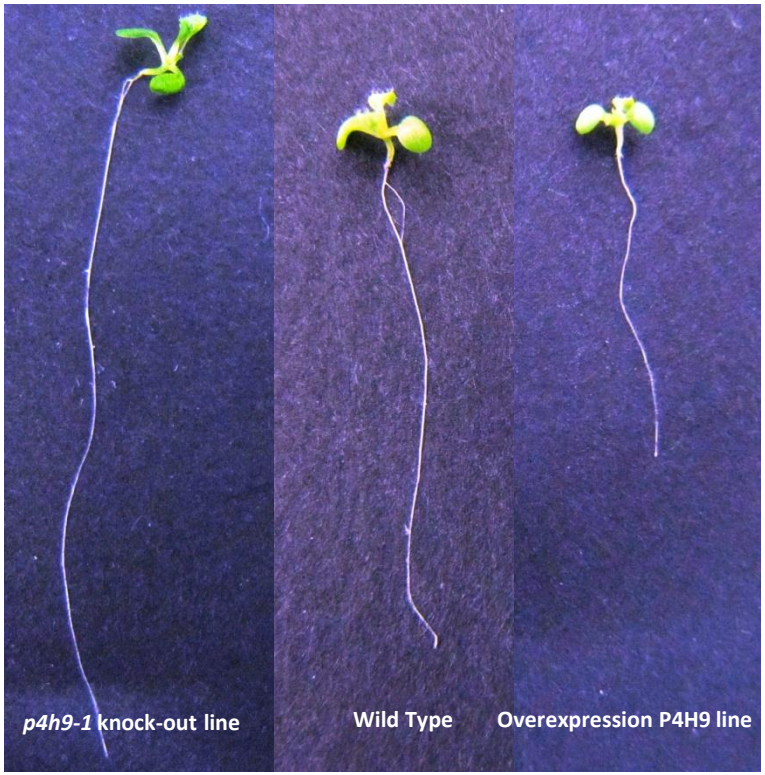


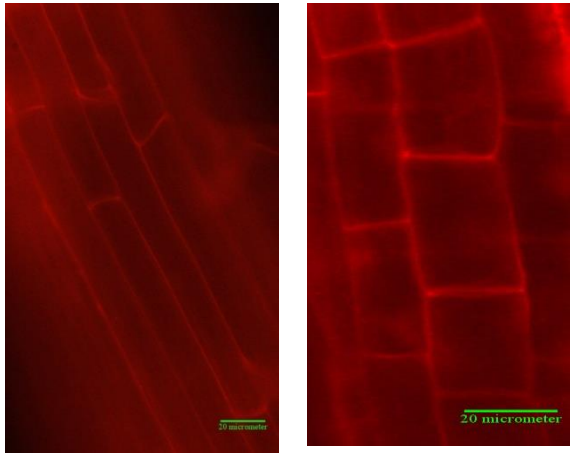
Extensin

P4H involvement in cell elongation

(Velasquez et al., Science, 2011)

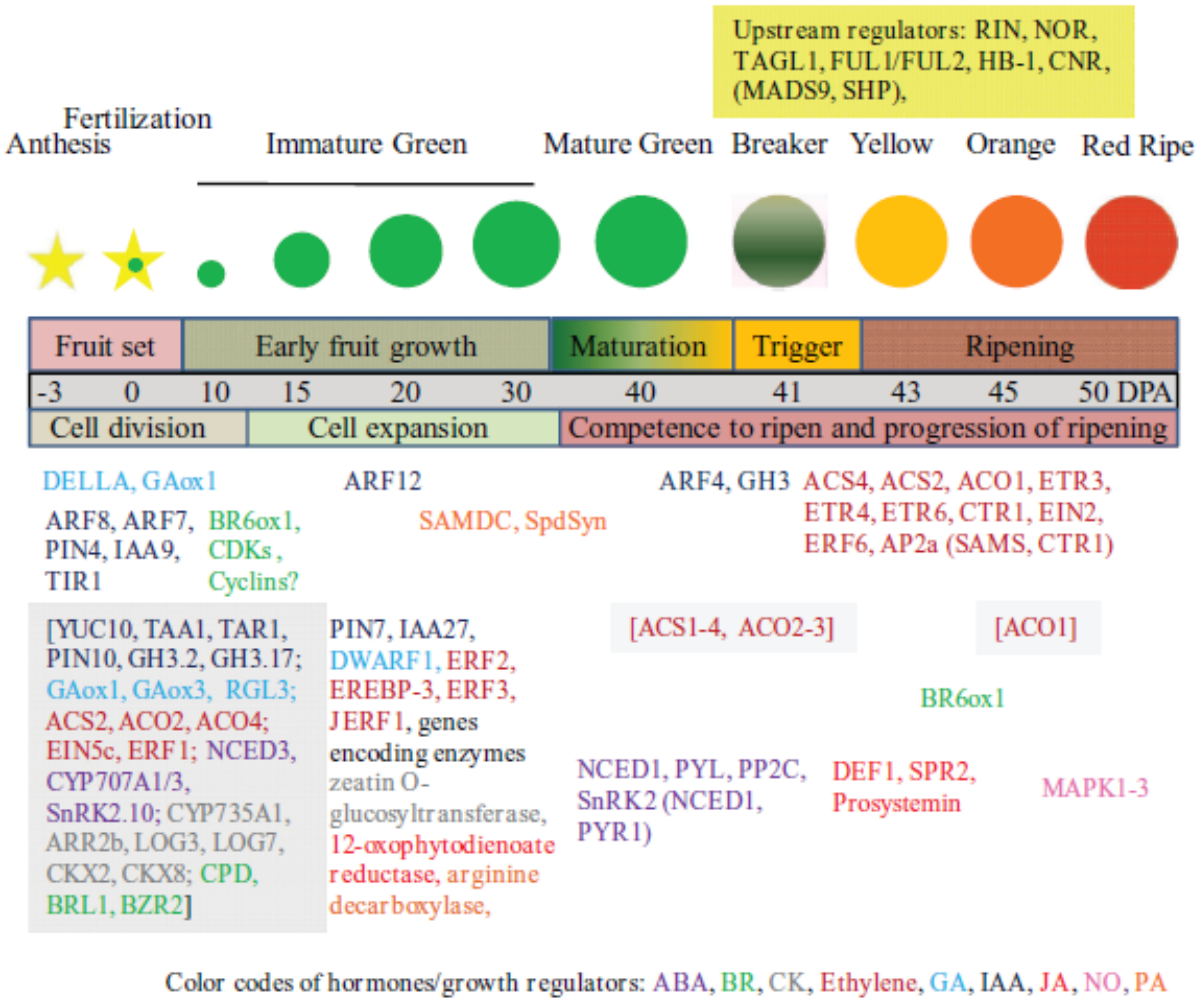
Arabidopsis P4H9 is involved in root length determination by regulating cell elongation





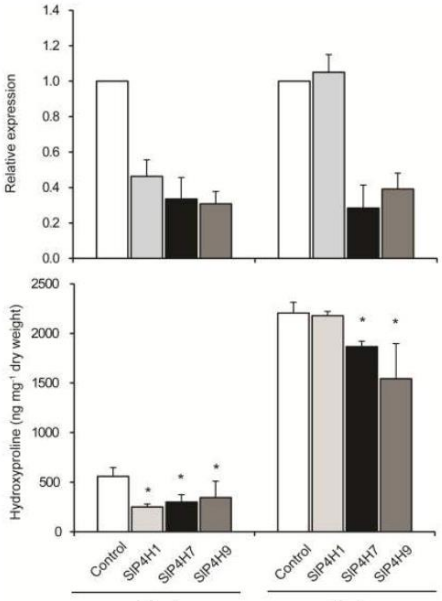
P4h9-1

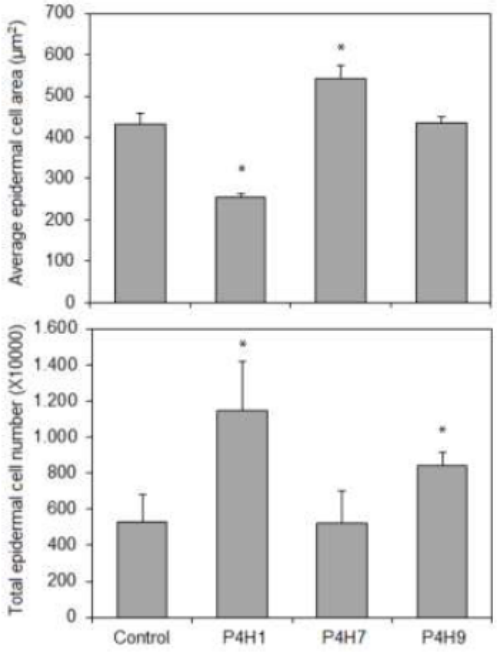
wt

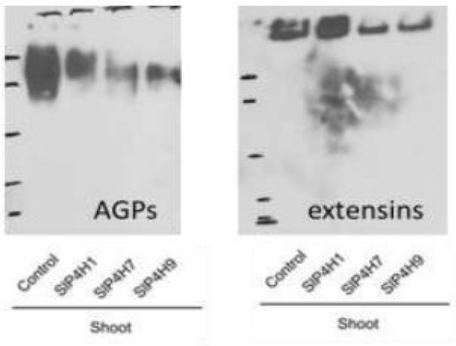


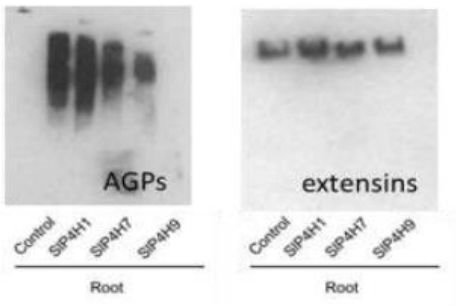
(Kumar et al. 2014)

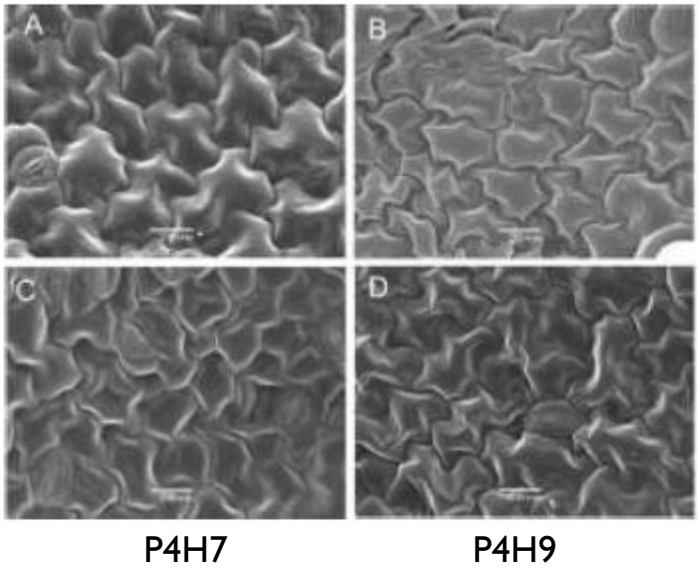
VIGS suppression of 3 Tomato P4Hs



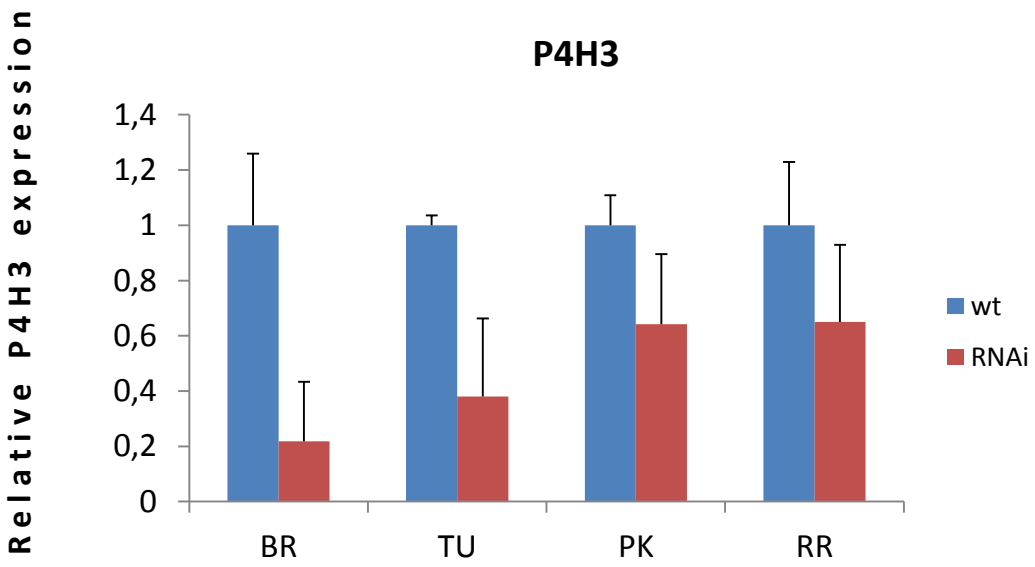




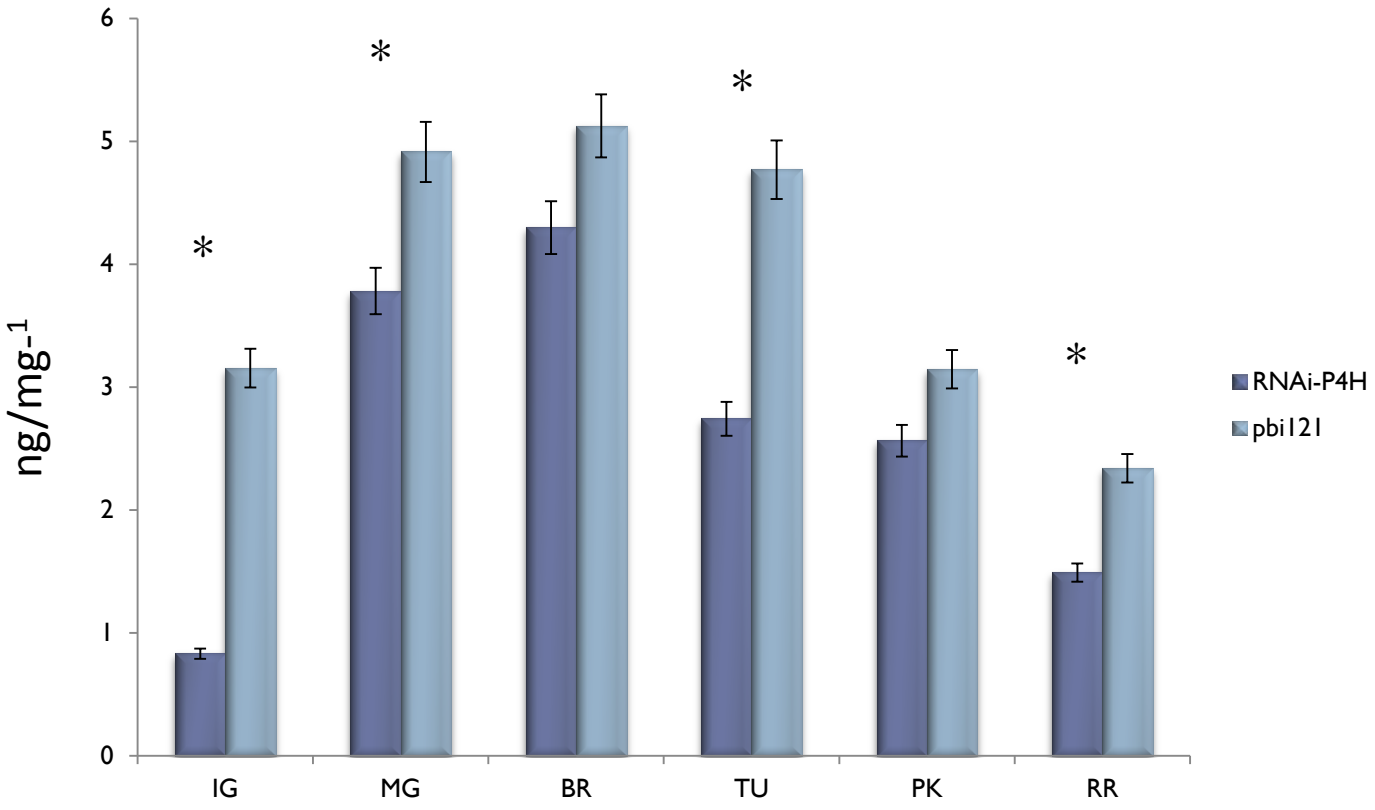




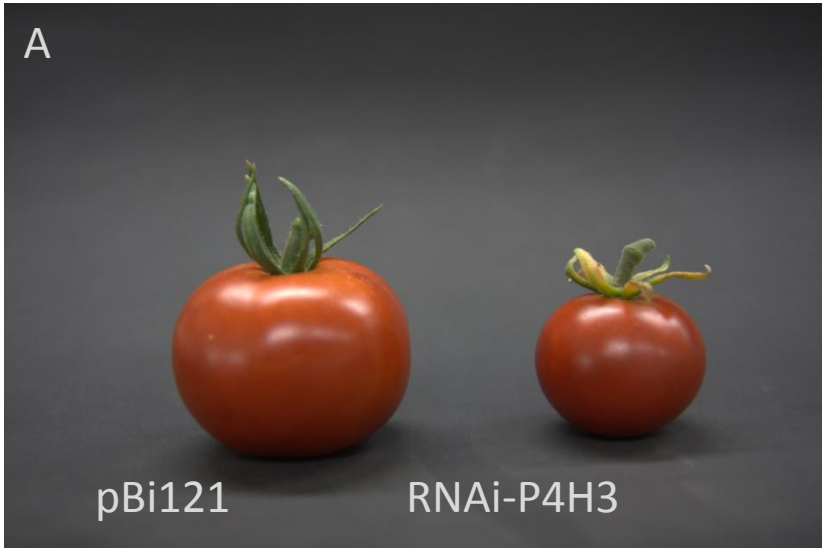
Fragostefanakis et a.

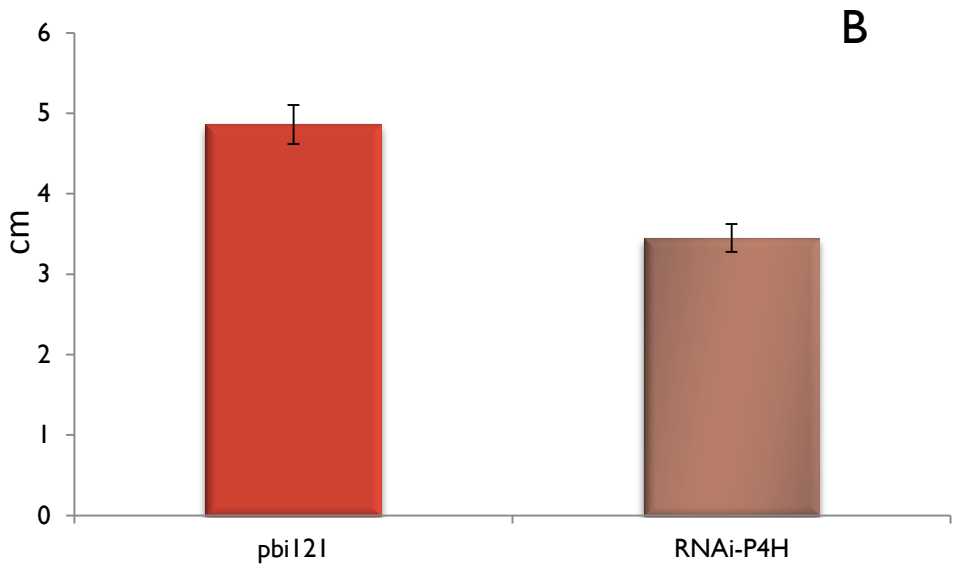


Relative expression of P4H3 in RNAi – P4H3 line #7



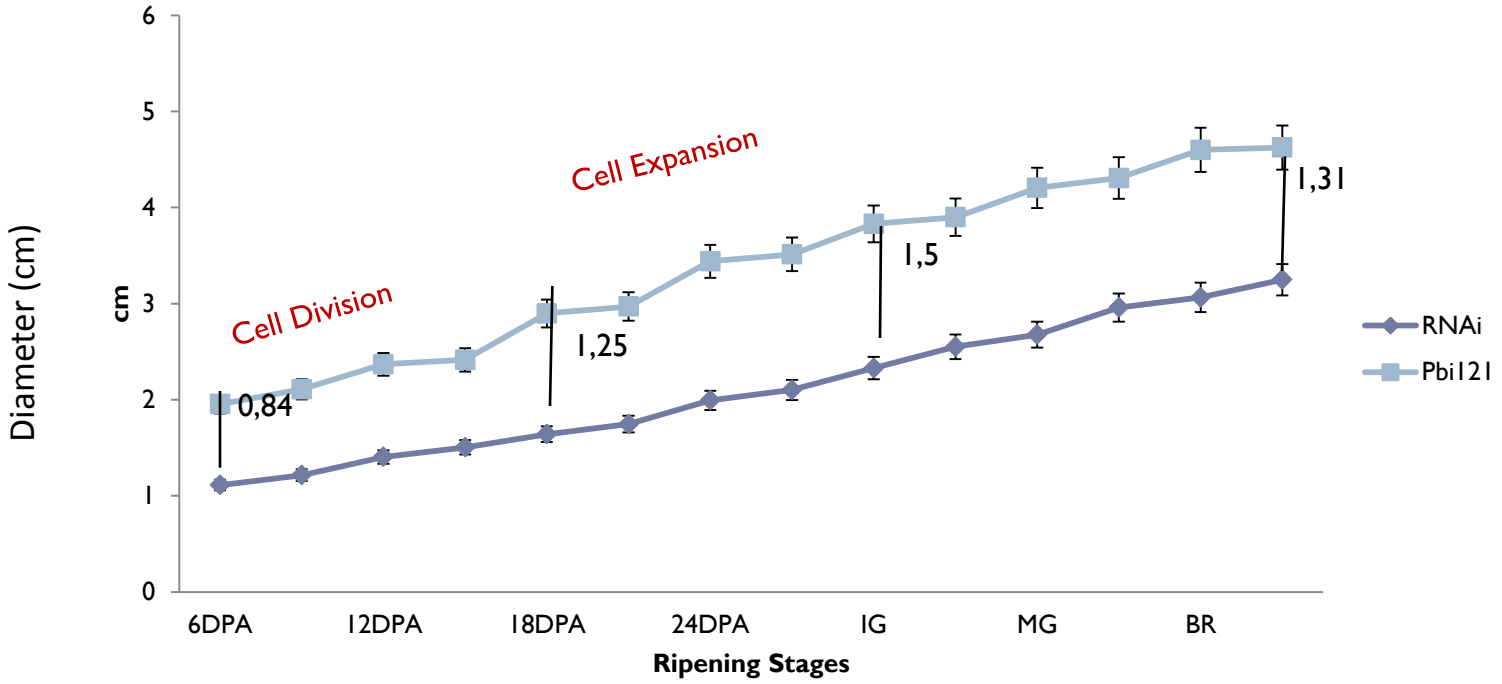
Hydroxyproline content during fruit ripening





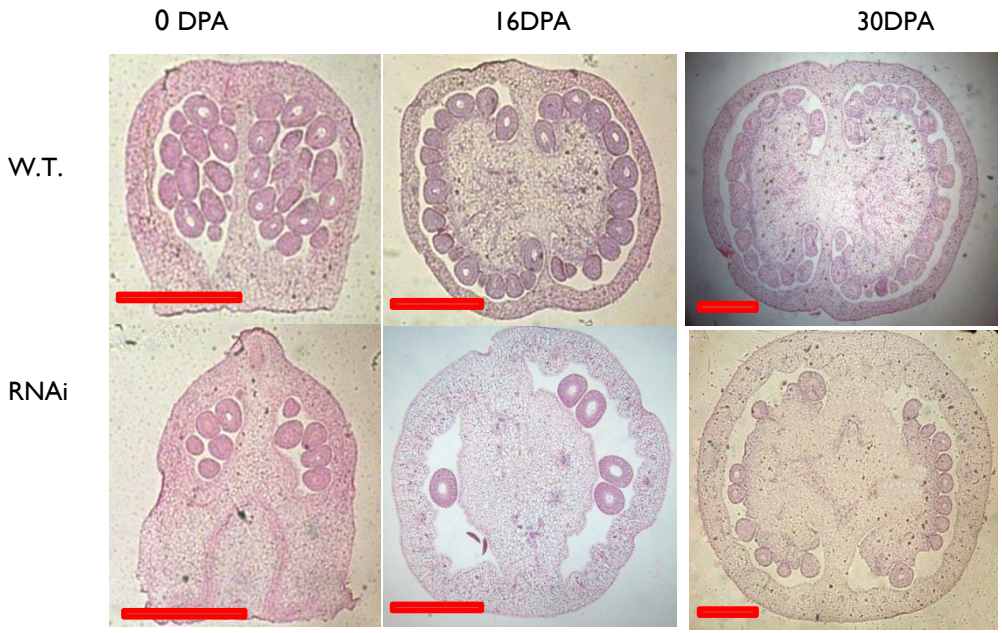
Fruit Diameter (cm) at BK

(A) Fruit diameter determined at the full-ripe stage (BK + 10).
(B) The data represent the mean values of line #7
All nine independent transformants gave similar results



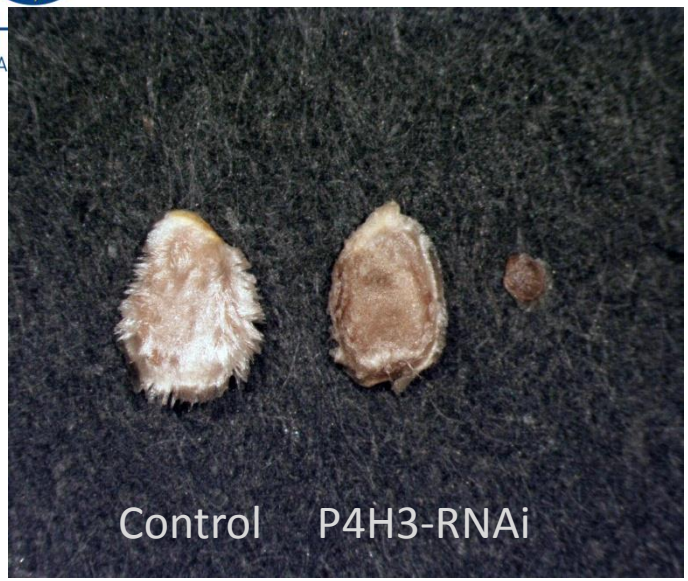
Fruit diameter was recorded every two days, for the first six inflorescences of 50 plants from 9 independent transformants

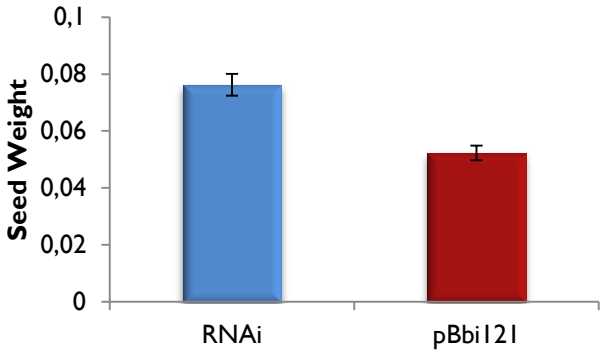
TRANSVERSE SECTIONS OF TOMATO FRUITS

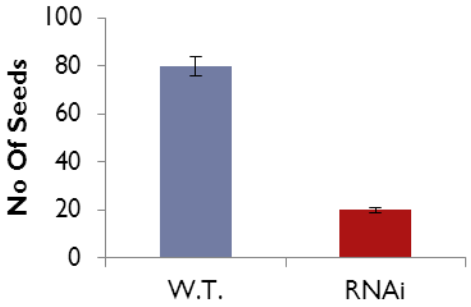


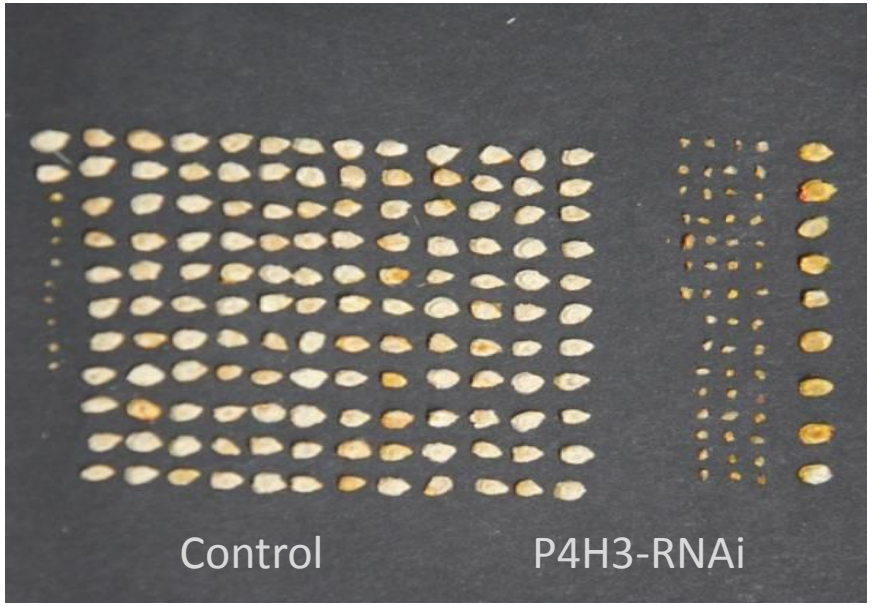
A) From left to right: Transverse sections of day 1 PA. Transverse section of 16 and 30 days fruits after pollination. Effect of RNAi gene silencing of *SlP4H3* on cell area (**B**) and total number of pericarp cells (**C**). *Error bars* represent SD and asterisk significant different. Area of measurement 250μm^2 , Bar 400 μm

CHARACTERISTICS OF SEEDS



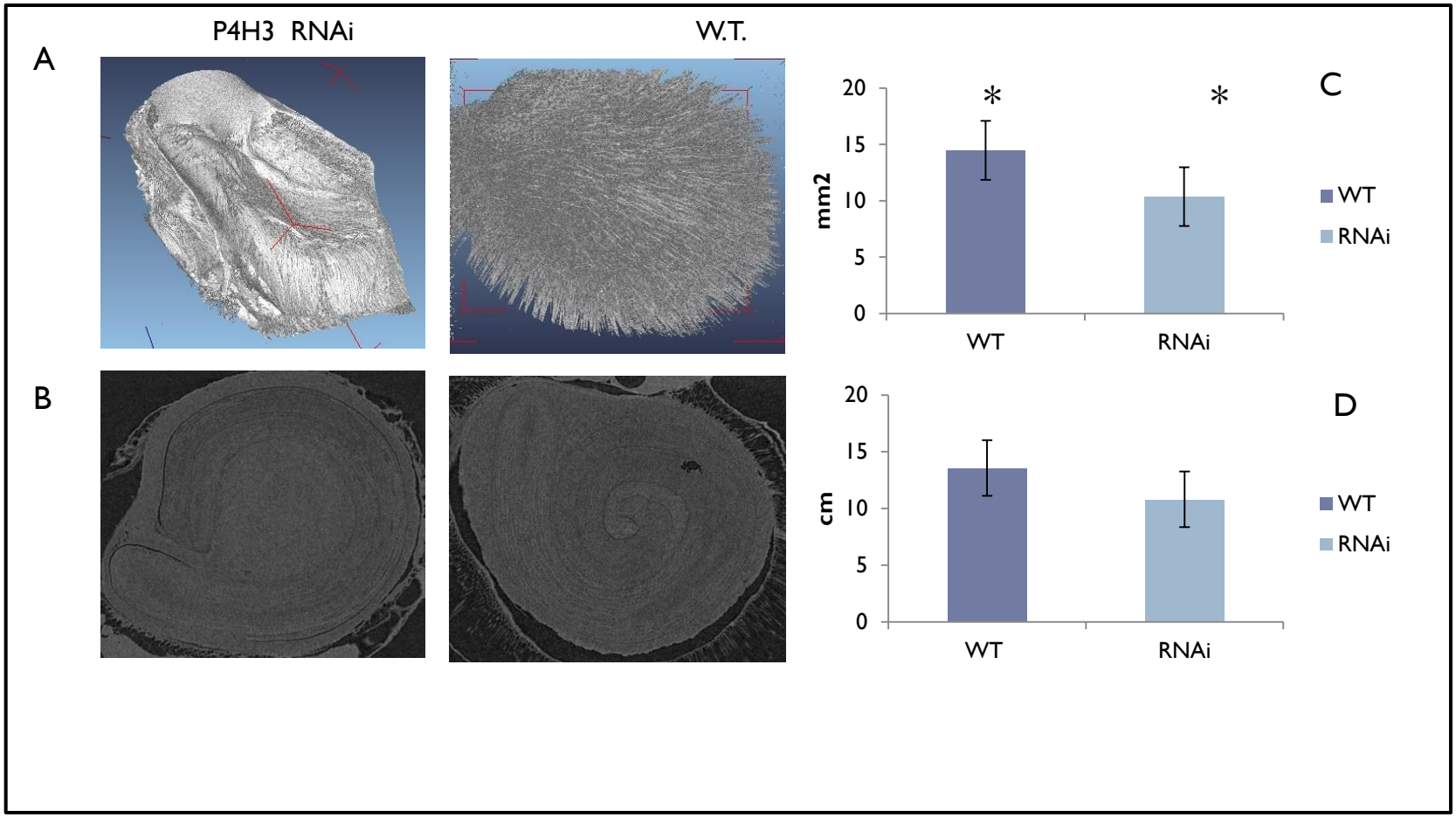






Seed weight of SlP4H RNAi and pBi121 (Control). The data represent the mean values obtained from 8 plants of three independent SlP4H RNAi transgenic lines and 21 pBi121 (control) plants. Statistical analyses were realized using the Student's test $P<0.005$;

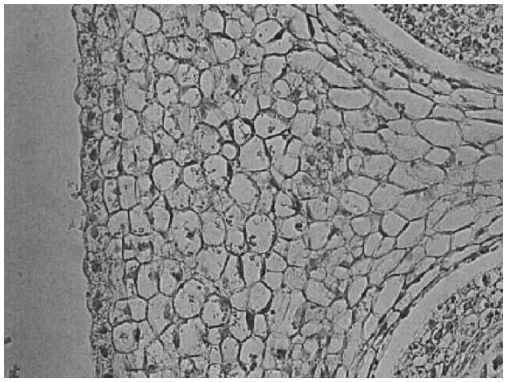
Micro Computer Tomography Results



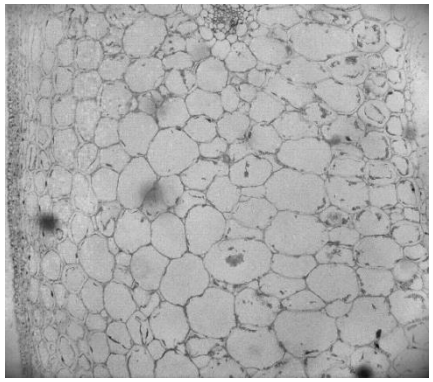
Images captured by Micro Computer Tomographer. A) Outer image of P4H3 RNAi and W.T. seeds. B) Cross-section of P4H3 RNAi and W.T seeds. Image shows the development of radicle, which was used to calculate the average radicle area (C) and length (D) among six seeds per line that were examined. Significant differences based on Student-t test ($P < 0.05$) are indicated using asterisk.

Slides from Microscope

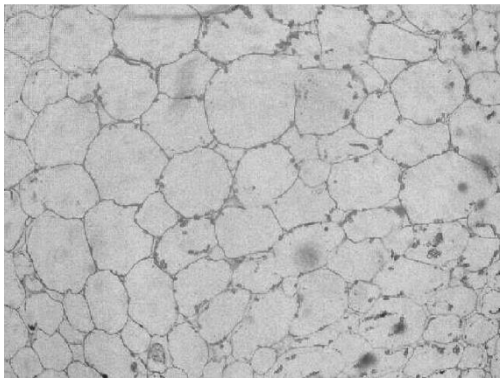
Wild Type



Day1

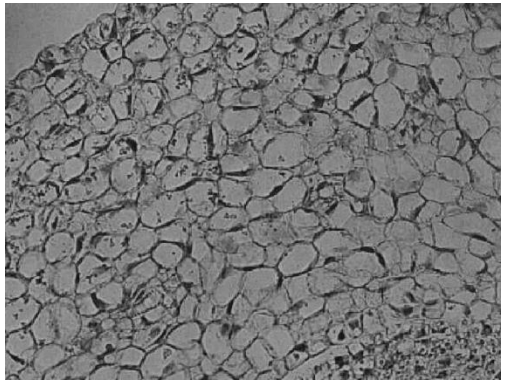


Day16

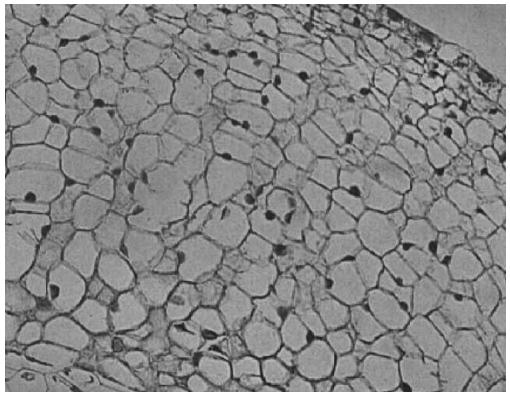


Day30

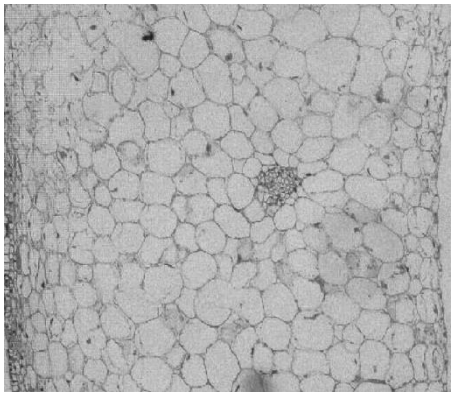
RNAi



Day1

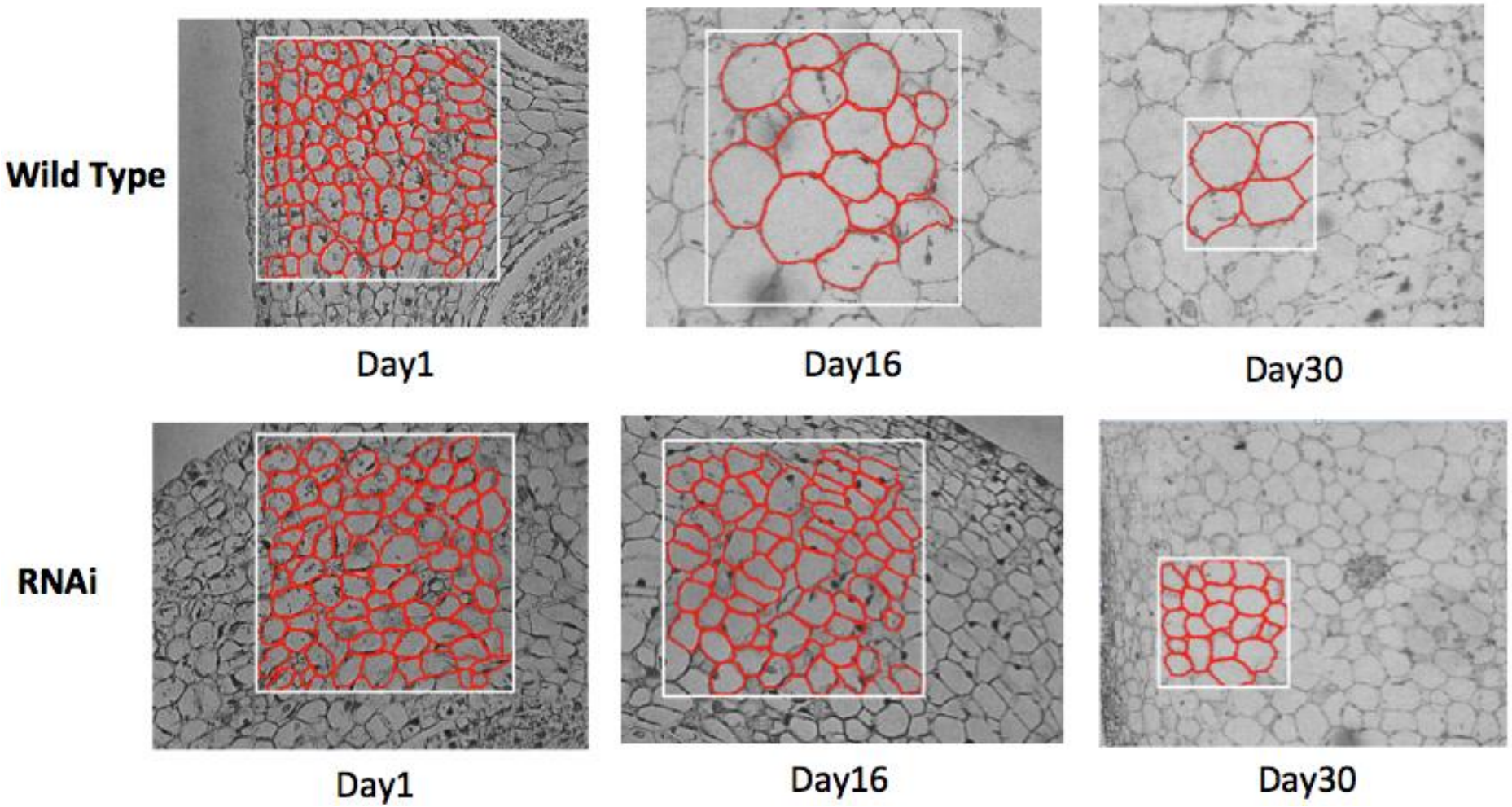


Day16



Day30

- **Image segmentation**: We created a semi-automatic algorithm for cell contour extraction using the Image Processing Toolbox from



- **Proportional size of boxes has been selected according on each image resolution.**

Morphological analysis-Number of cells

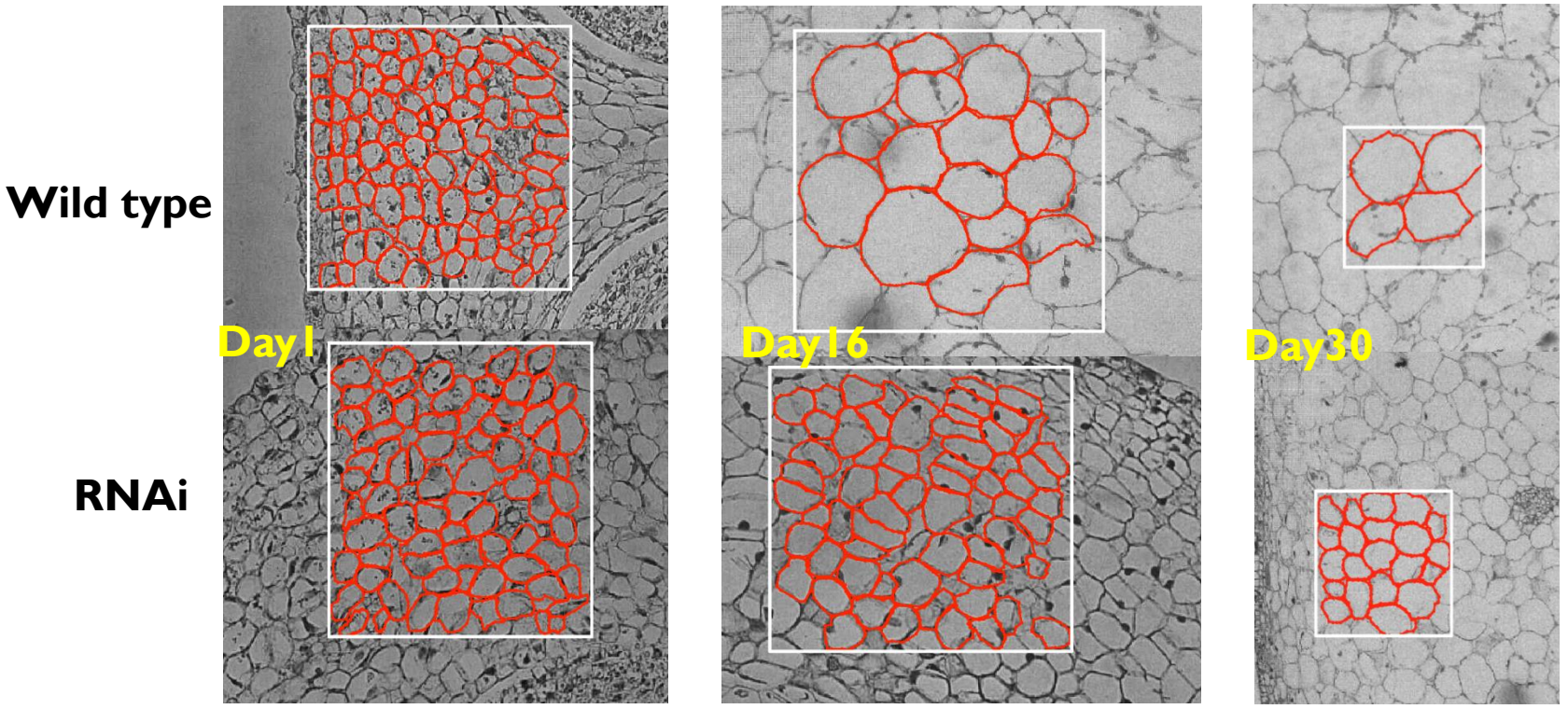
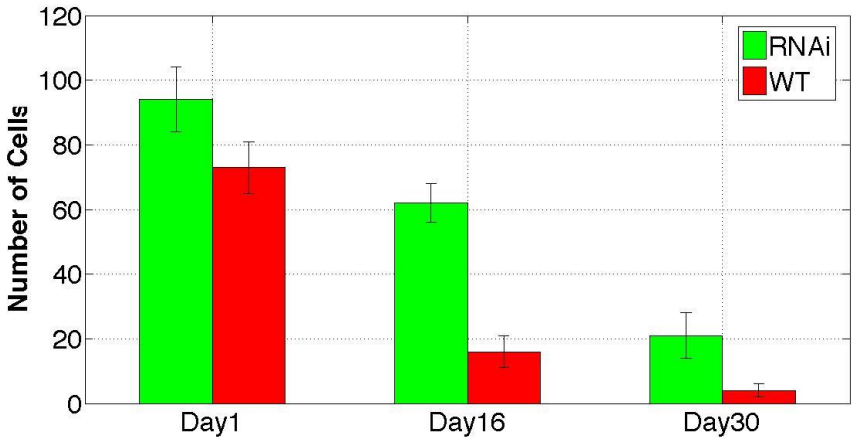
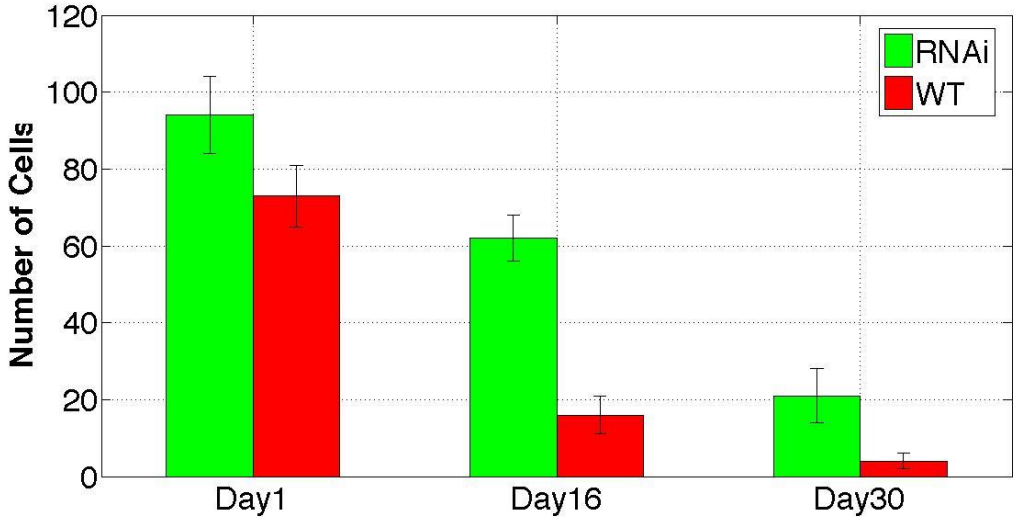


Figure1 : *Number of cell per microscope slide.* We observe that there is a decrease in the number of cells during the plant growth.



Relation between cell size and the number of cells.



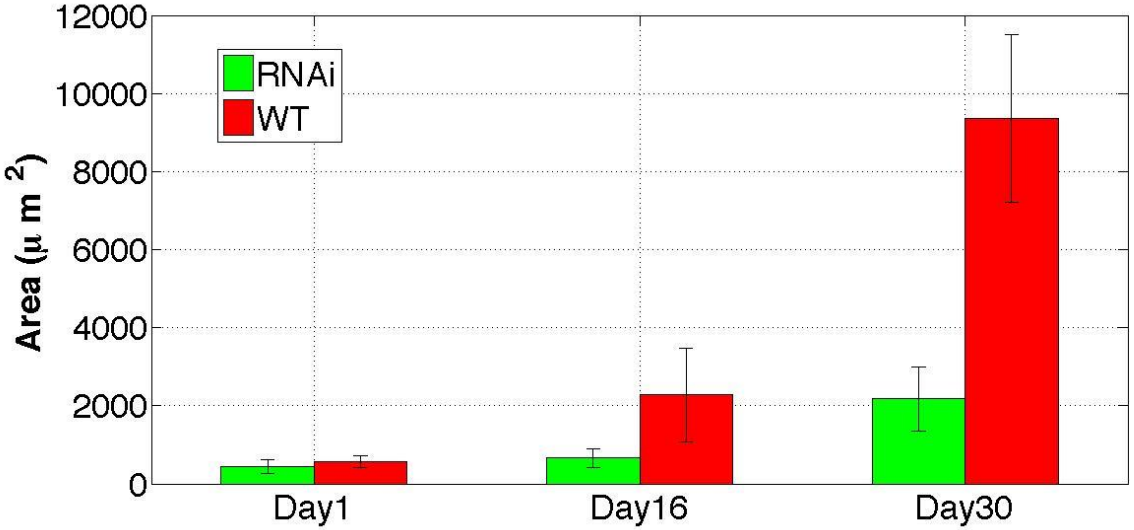
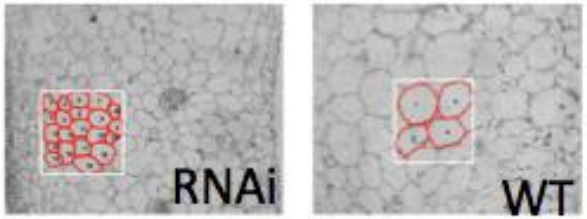
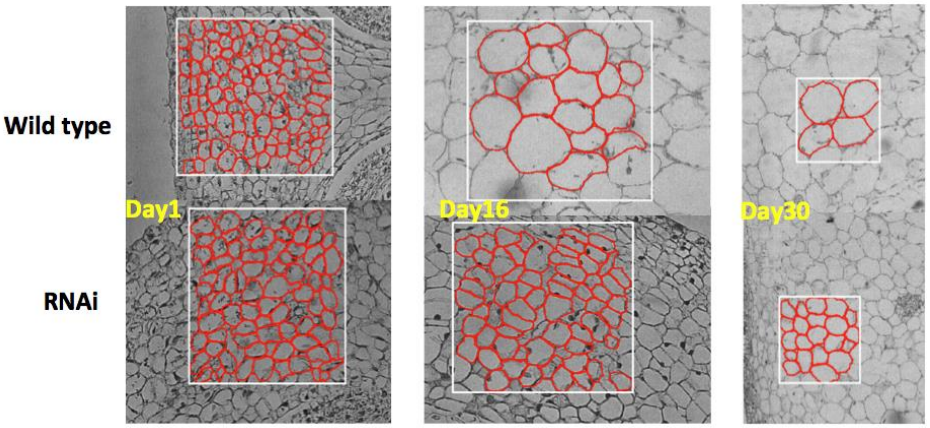


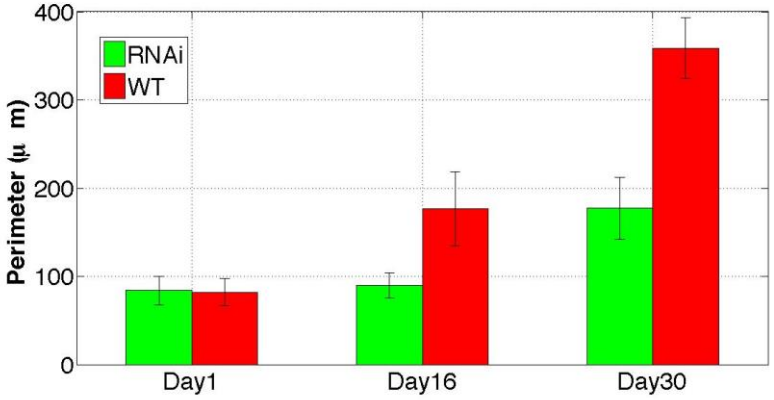
Figure 2: *Average cell size per microscope slide.* We observe that the average cell size on the WD it is bigger than those of RNAi, especially on the 30th day.

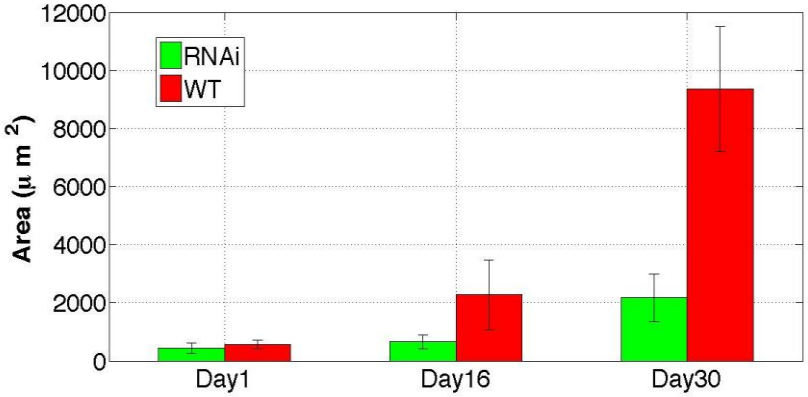


- **General Comment:**
  It is obvious that there is an inverse relation between the cell size and the number of cells, during the plants growth.

Morphological analysis-Perimeter of the cells

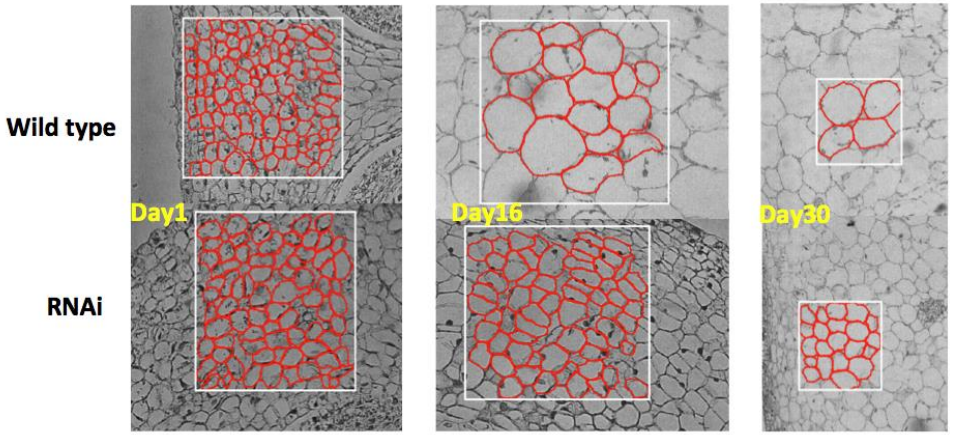






- We observe that the average perimeter of each cell increases significantly, while the cell size increases.
- The average perimeter of the cells from RNAi seems to remain stable between the 1st and the 16th day; whereas the perimeter of the cells from WT is doubled.
- Our numerical findings show that there is 50% increase in the perimeter of the average cell between the RNAi and WT, during the 16th and the 30th day respectively.

Morphological analysis-Circularity of the cells



Circularity is a dimensionless parameter that reflects the ratio of cell size to the cell boundary perimeter. For circularity, a value of one represents a perfect circle, while values approaching zero have a more linear or complex structure.

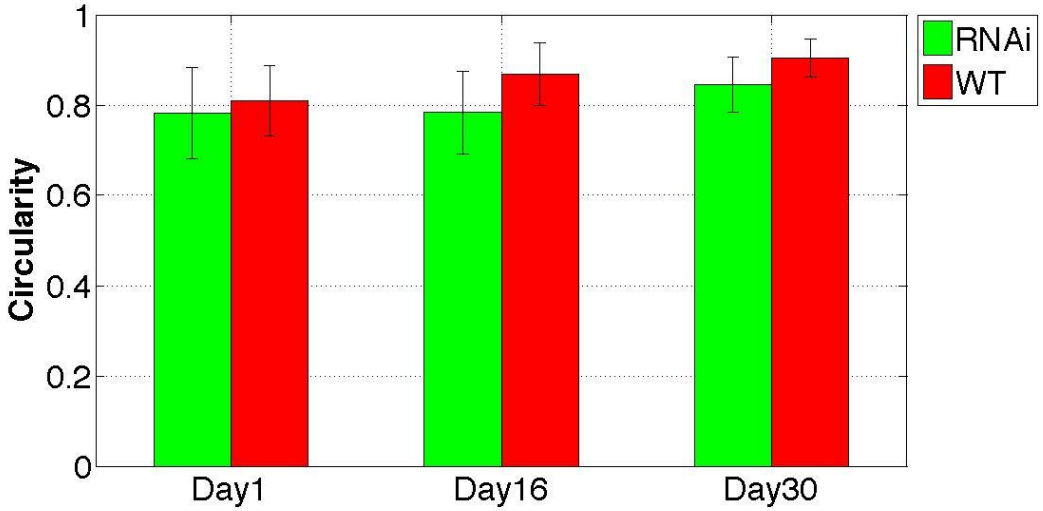
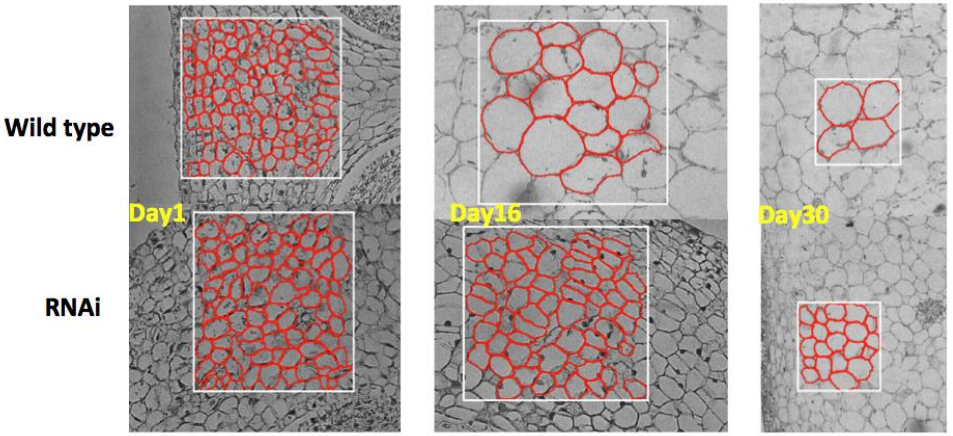


Figure: *Circularity of an average cell.* We observe that the cells from the RNAi preserve their shape while the WT cells tend to become more circular after the 16th day.

Morphological analysis-Solidity of the cells



Solidity describes the extent to which the shape is convex or concave. When its values approaching one correspond to rounder, more solid cells.

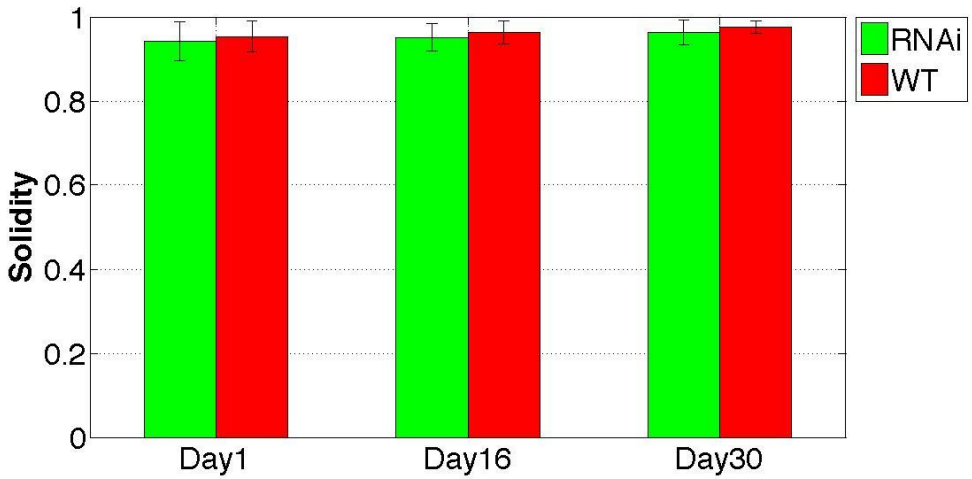
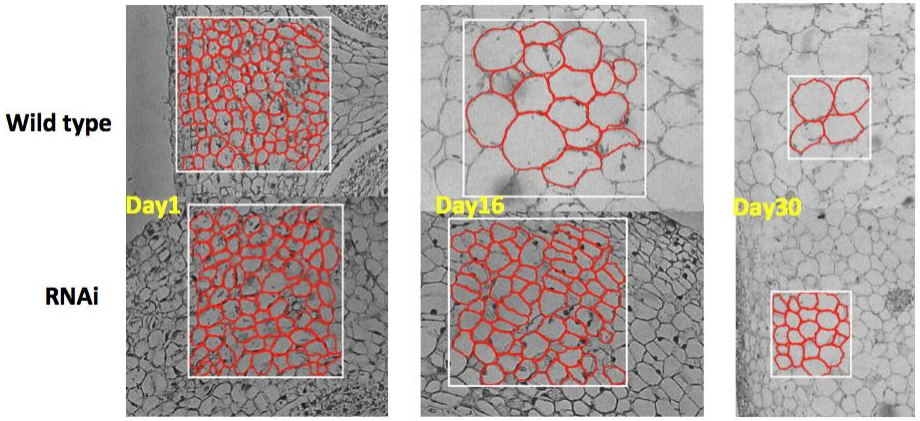


Figure: *Solidity of an average cell.* We observe that both the cells from the RNAi and WT have similar solidity values, during the fruit development. However, WT cells appear to be slightly more solid than the RNAi.

Morphological analysis-Rectangularity of the cells



Rectangularity has been designed to evaluate numerically how much the shape considered differs from a perfect rectangle. The range of its values is the interval [0,1] and attain the value 1 only for perfect rectangle with a desired edge ratio.

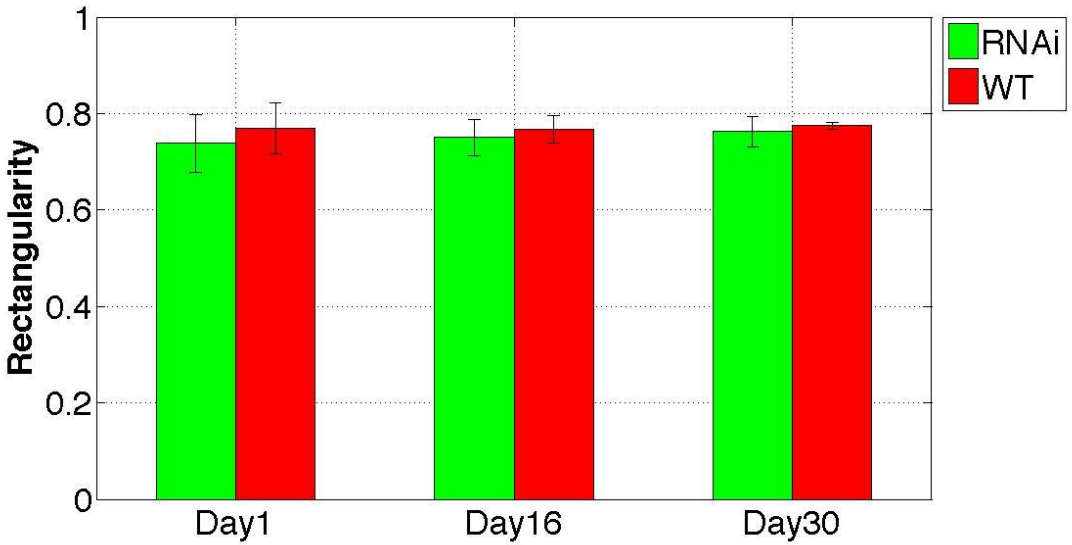
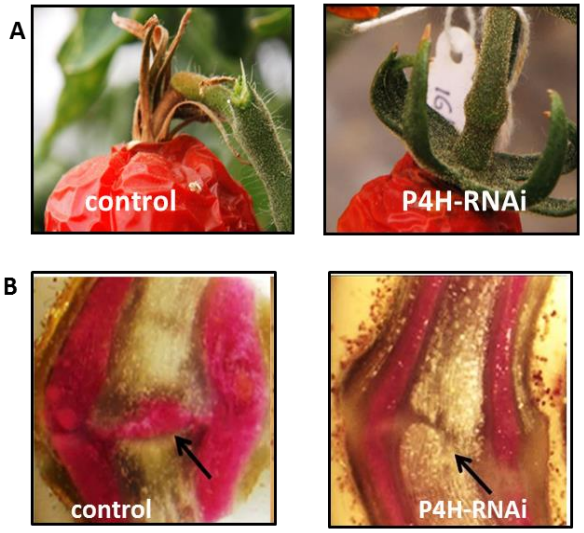


Figure: *Rectangularity of an average cell.* We observe that both the cells from the RNAi and WT have similar rectangularity.

Abscission related traits of the P4H-RNAi lines

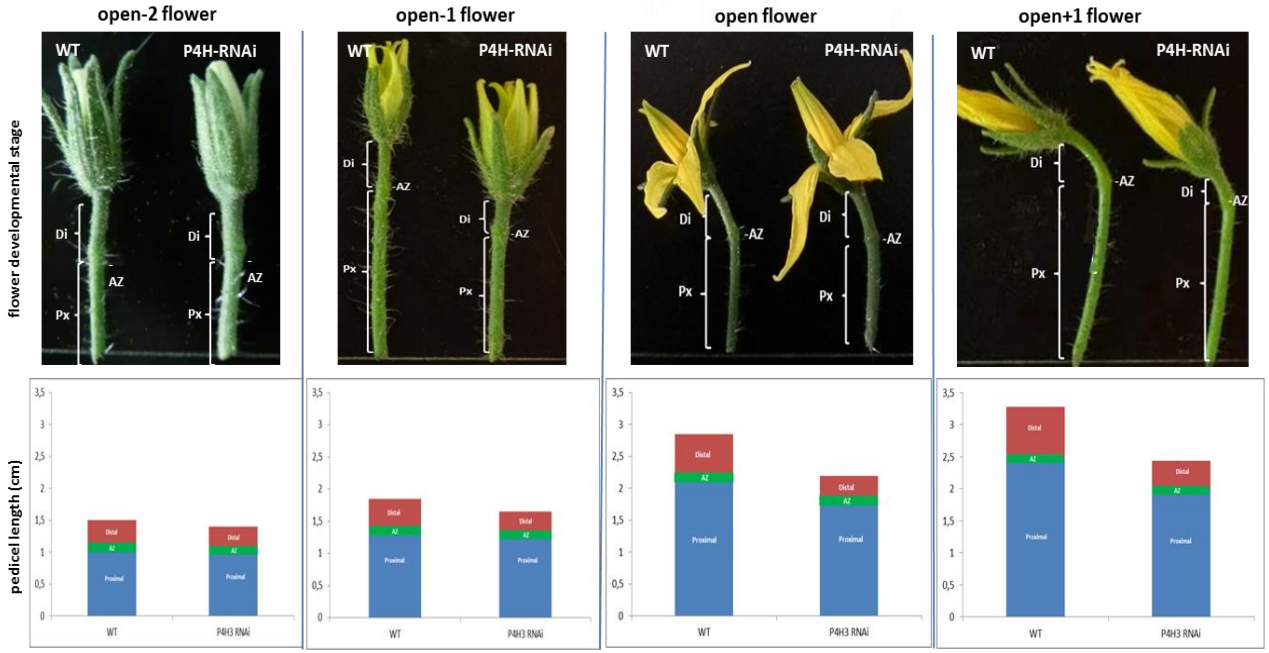
Delayed fruit abscission



Phloroglucinol (stains lignin)

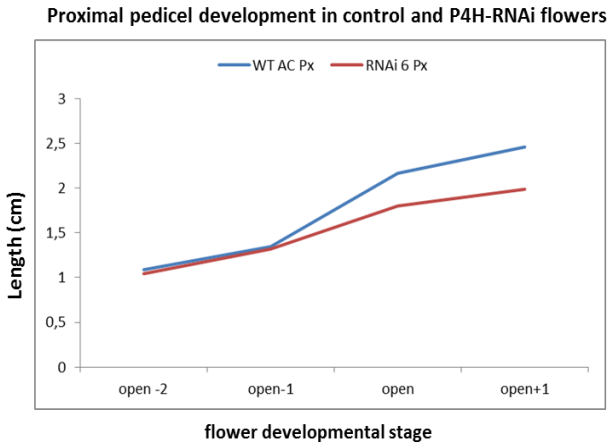
A) Delayed abscission of the P4H-RNAi fruits. B) Histochemical staining of pedicel abscission zones reveals a defect in lignin accumulation in the abscission zone of the RNAi line, indicating deficiencies in the normal progression of the abscission zone development.

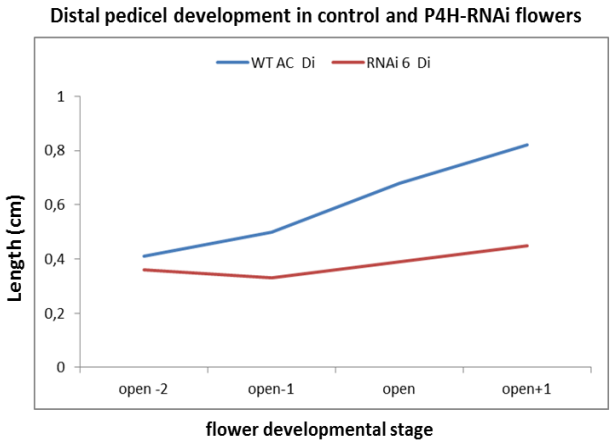
Flower pedicel development

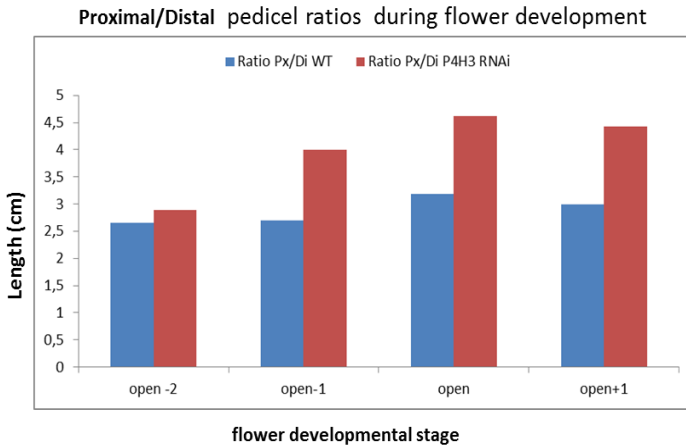


Development of flower pedicel is altered in P4H-RNAi flowers during flower growth. The distal section of the pedicel (Di) remains shorter in the mutant genotype.

Variance of proximal/distal pedicel ratios during flower development



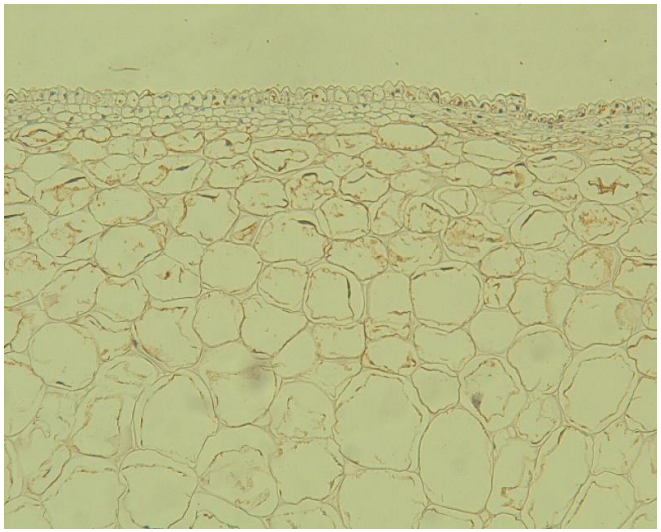




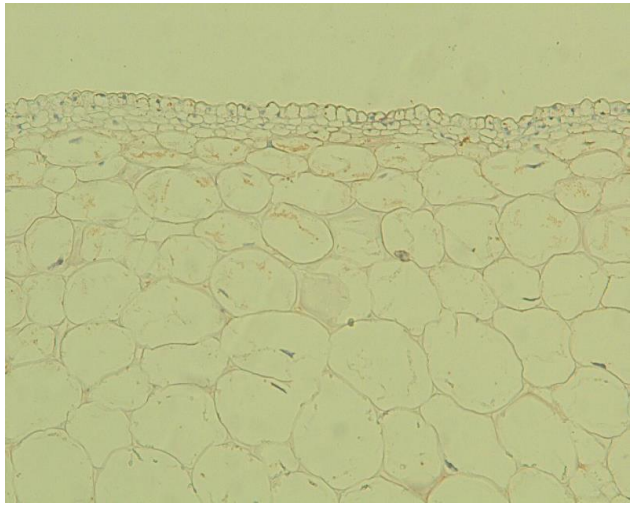
To summarize:

- ✧ There is a decrease in the number of cells both in the WT and RNAi, during the plant growth.
- ✧ The average cell size on the WD is bigger than this of RNAi.
- ✧ There is an inverse relation between the average cell size and the number of cells, throughout the plant development.
- ✧ Both the cells from RNAi and WT seems to maintain their morphology features. Whereas the WT cells seems to be more circular, especially on the 30^{th} day.
- ✧ Thus, inferring these cell shape parameters is an important and challenging task throughout the effort of unraveling the mechanism which is related in the tomato fruit development.

Immunolocalization of JIM8 epitopes



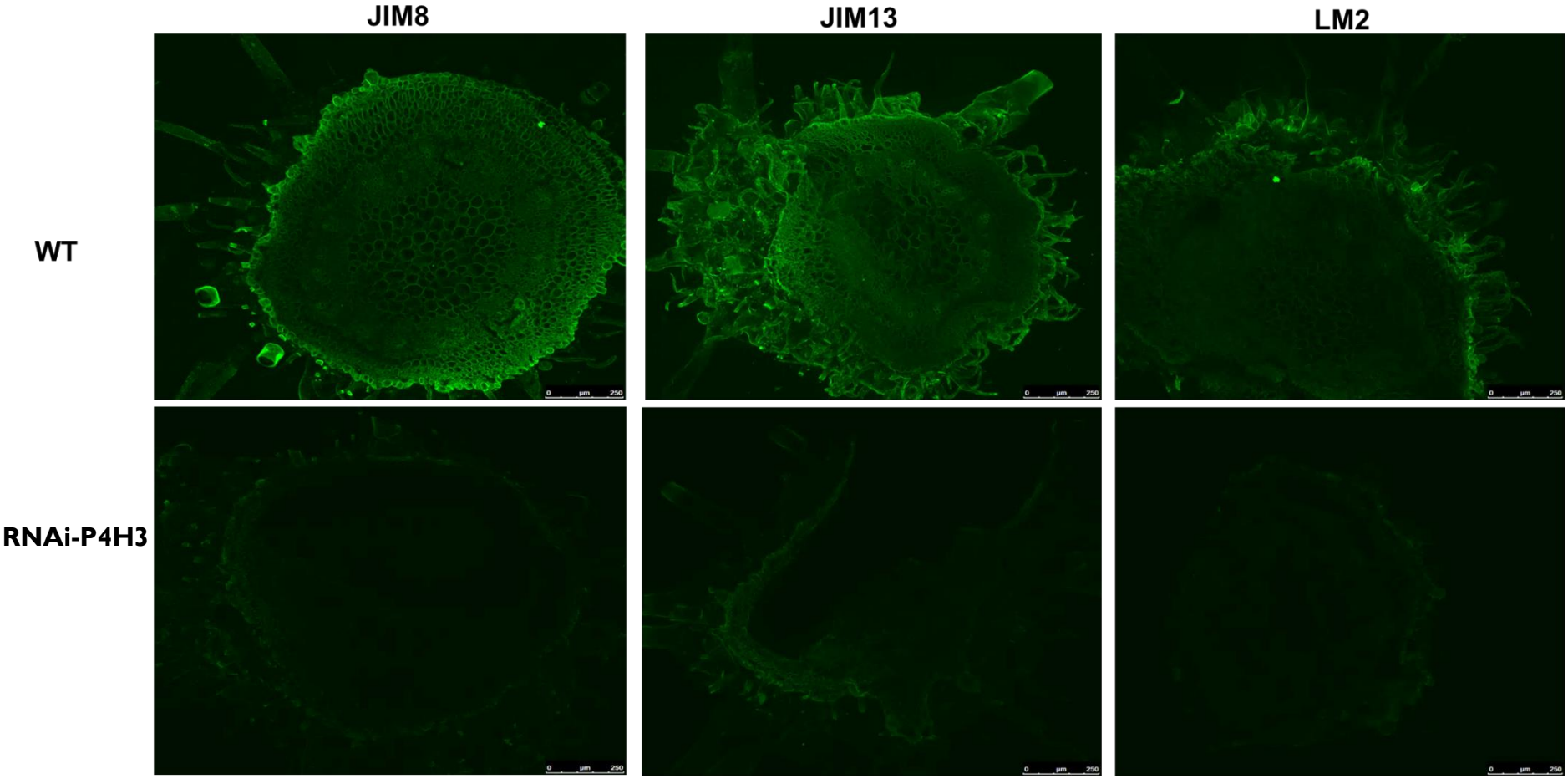
w.t.



P4H3 RNAi

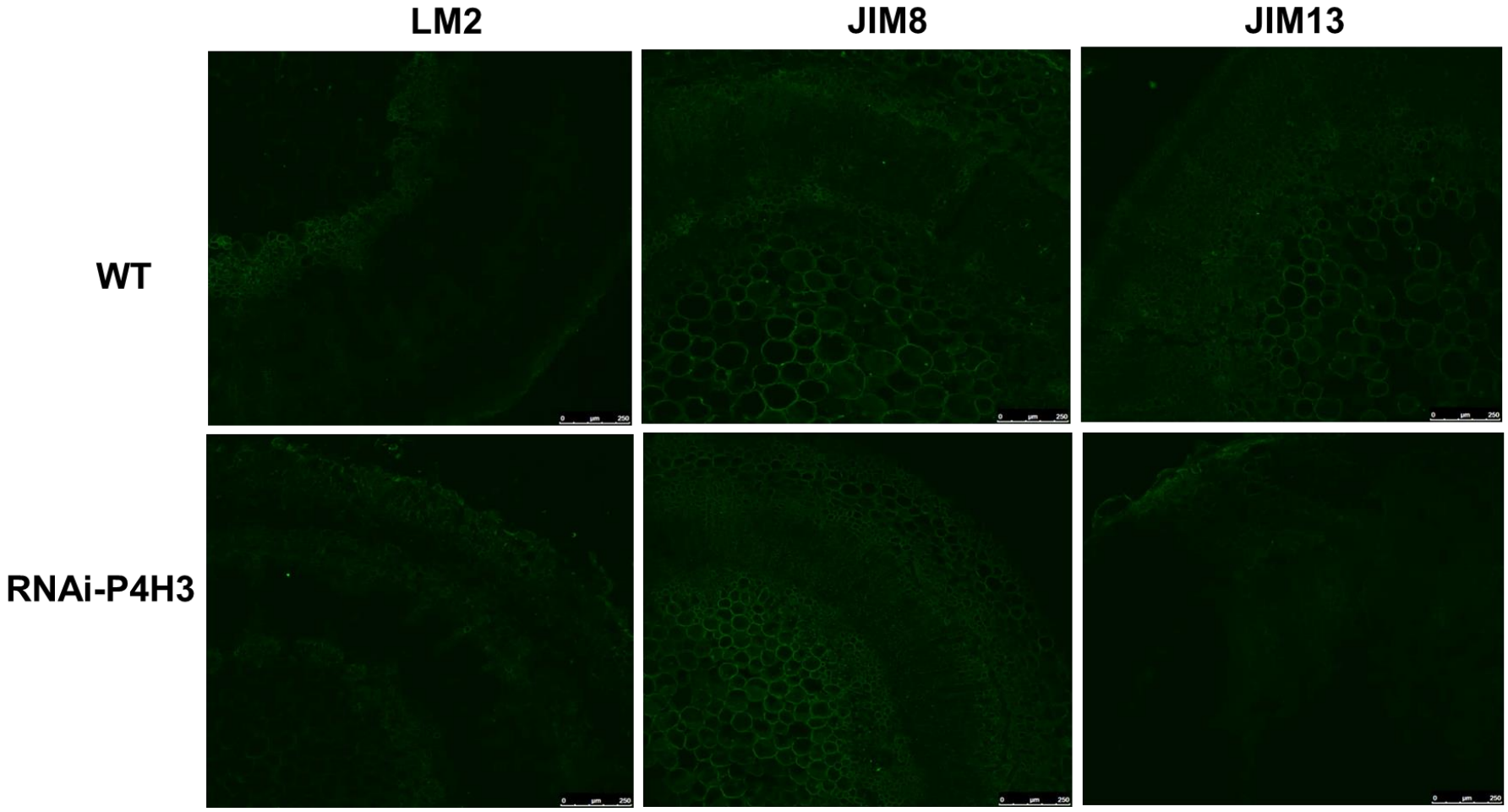
Immunihistochemistry (IHC) pericarp tissue of 30 days old fruit.
JIM 8 antibody was used for the detection of specific Arabinogalactan epitopes.

Flower AZ Immunolocalization

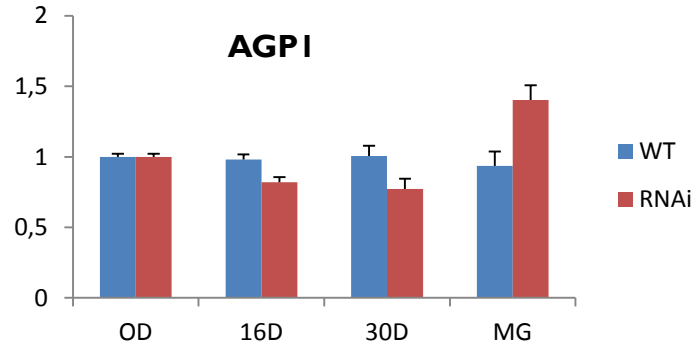


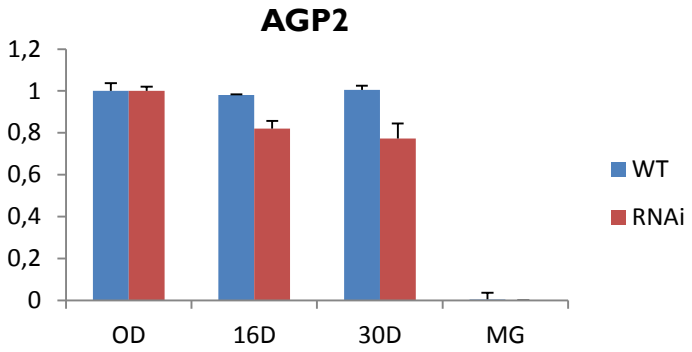
- AGPs specific antibodies were used in Flower Abscission zones. The immunolocalization analysis revealed significant differences of the RNAi-P4H3 lines when compared to the WT.

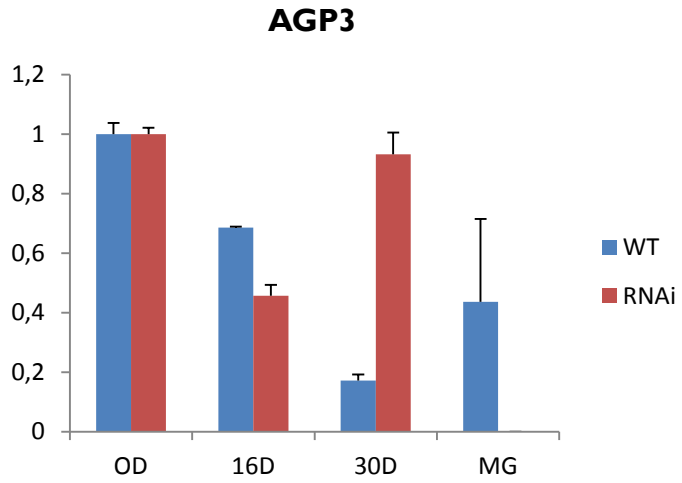
Fruit AZ Immunolocalization

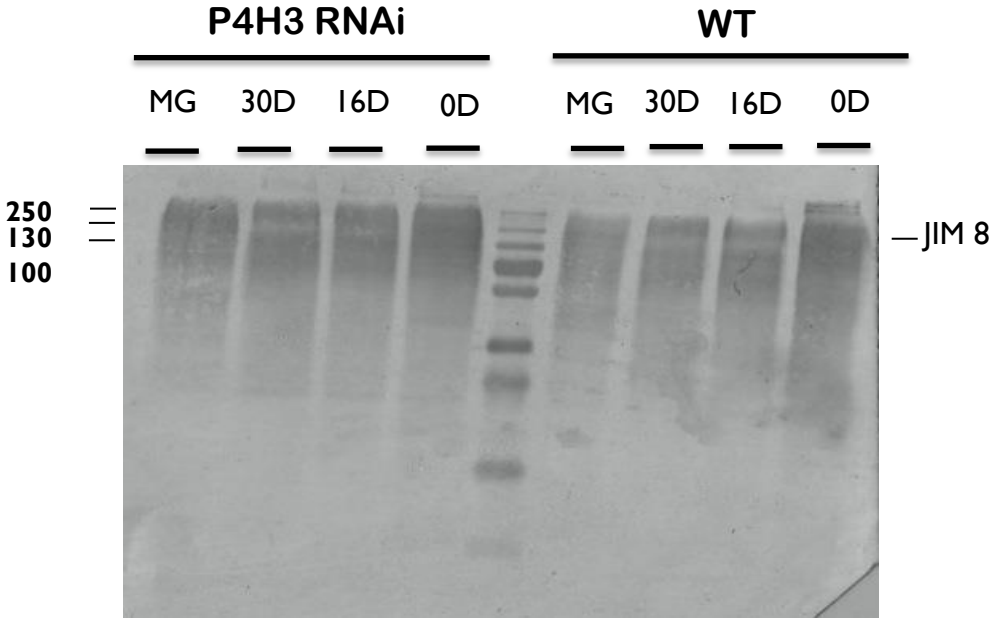


- AGPs specific antibodies were used in Fruit Abscission zones. The immunolocalization analysis revealed no significant differences of the RNAi-P4H3 lines when compared to the WT.

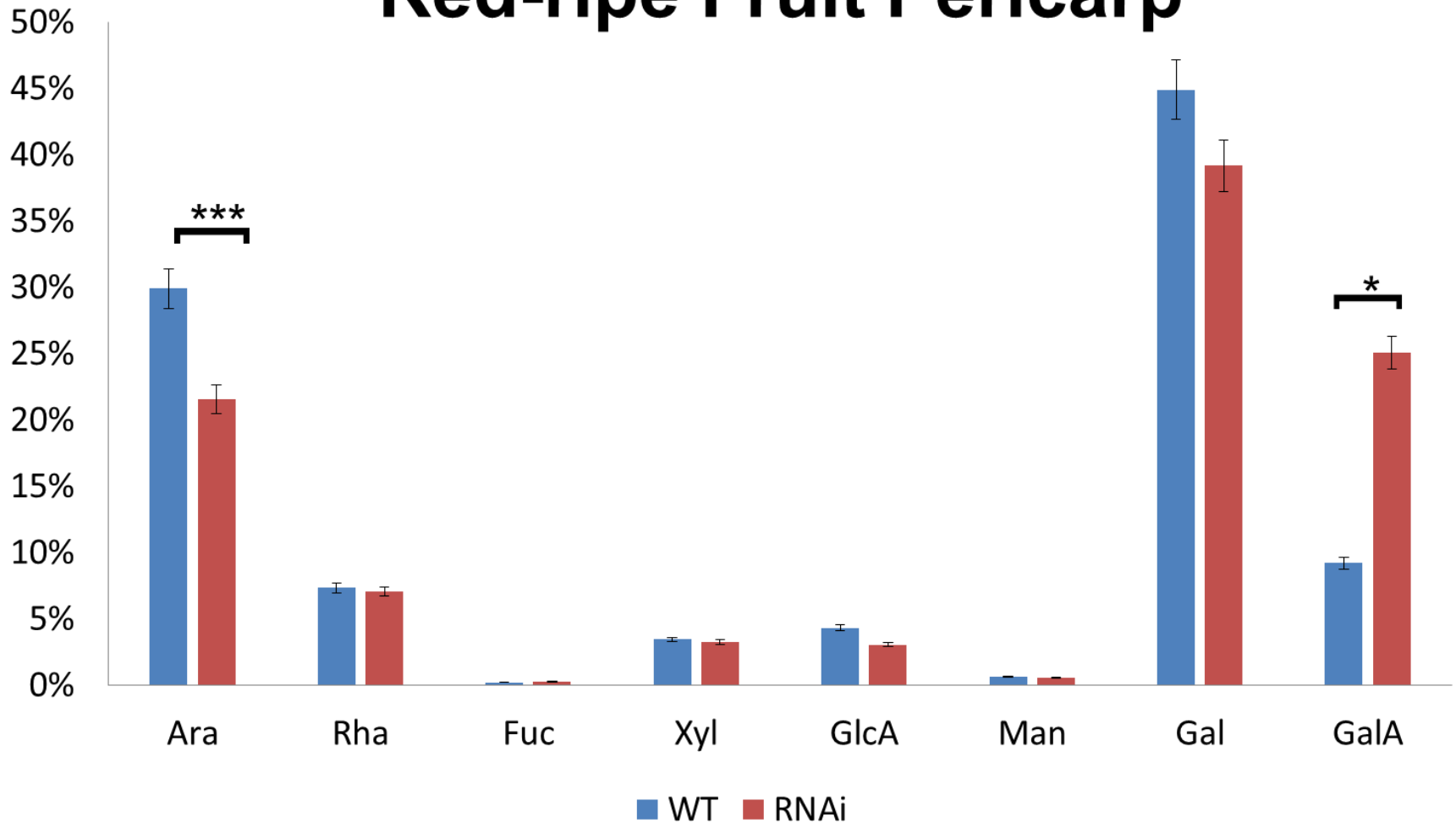

AGP1
2
1,5
1
0,5
0
OD
16D
30D
MG
WT
RNAi


AGP2
1,2
1
0,8
0,6
0,4
0,2
0
OD
16D
30D
MG
WT
RNAi


AGP3
1,2
1
0,8
0,6
0,4
0,2
0
OD
16D
30D
MG
WT
RNAi

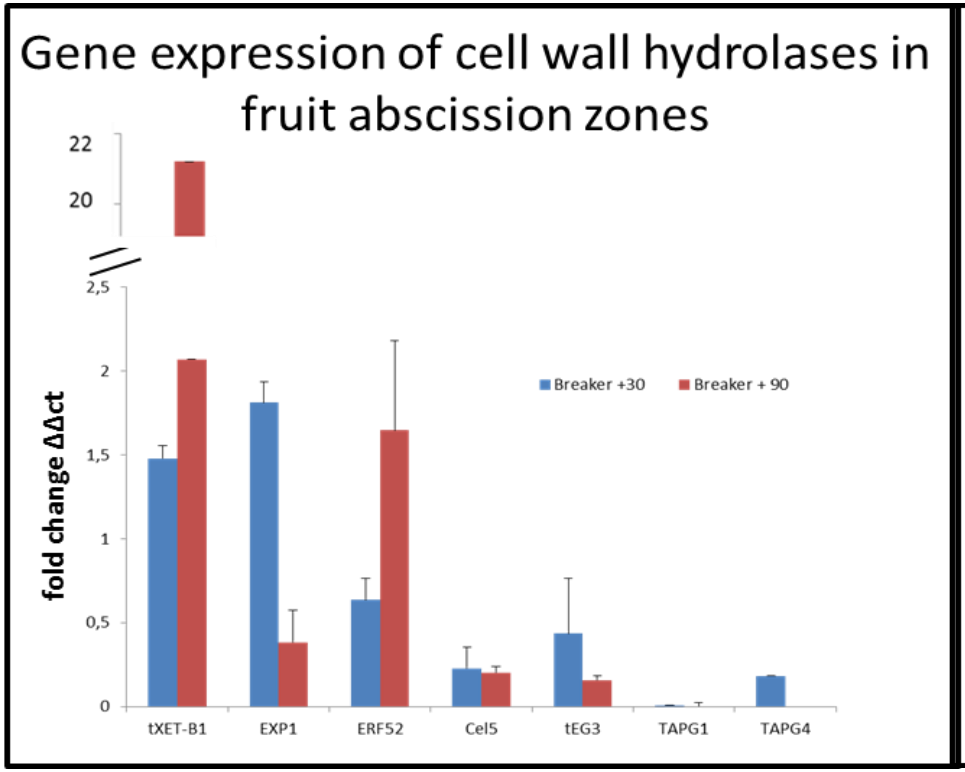

P4H3 RNAi
WT
MG
30D
16D
0D
MG
30D
16D
0D
250
130
100
JIM 8

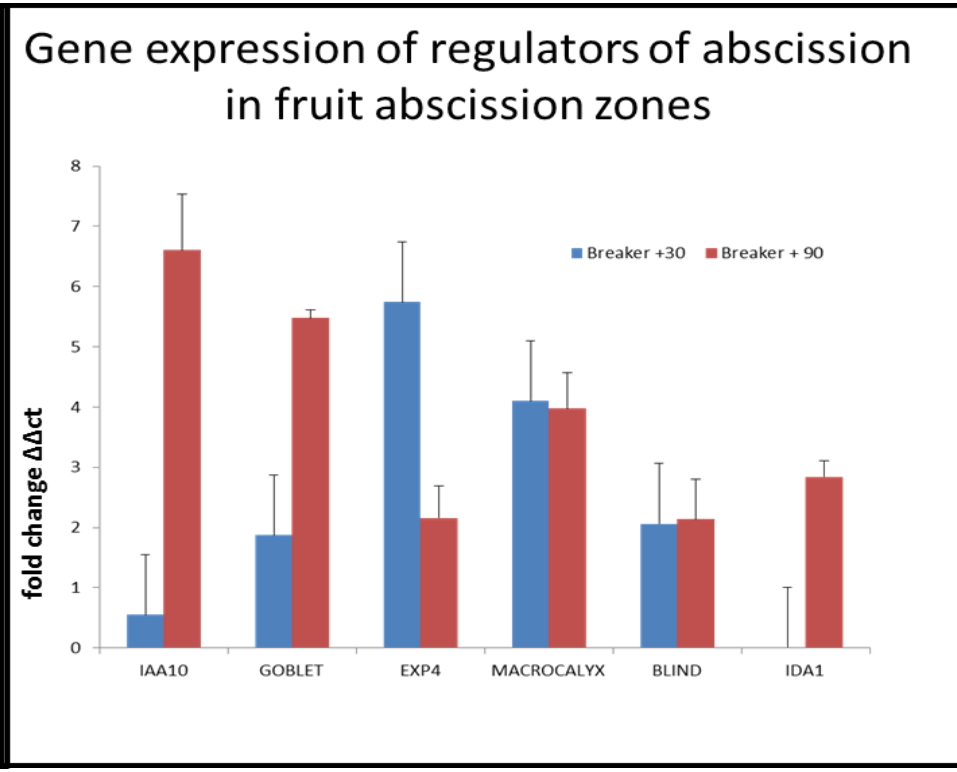
Sugar Content of Total AGPs in Red-ripe Fruit Pericarp



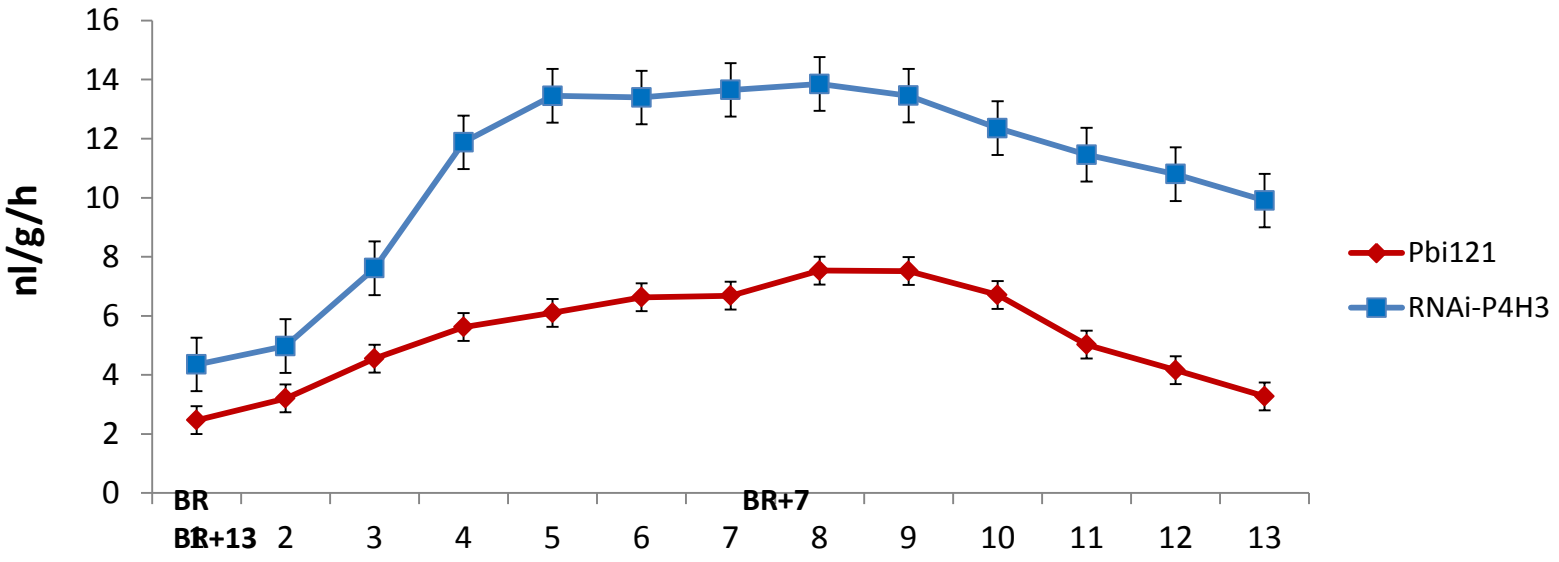
- GC-FID analysis revealed a significant decrease in Arabinose (Ara) and increase in Galacturonic (GalA) acid in the transgenic lines.

Gene Expression of cell wall hydrolases and abscission zone regulators

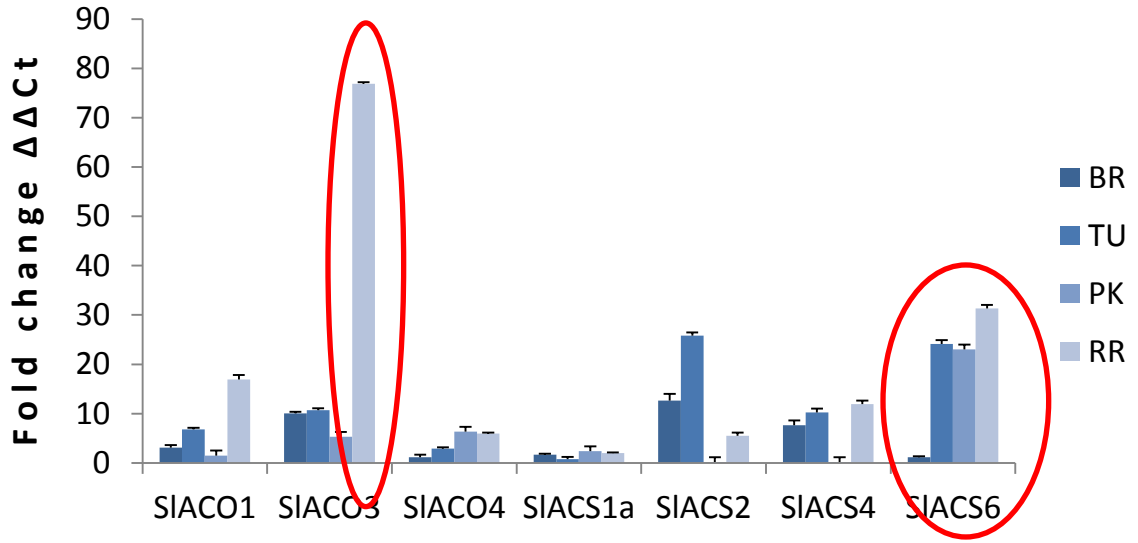




Ethylene production rate in P4H3 RNAi line



Expression of ethylene biosynthesis genes



Conclusions

- Silencing of a tomato P4H resulted in smaller fruits, decreased pericarp cell size and increased cell number, lower number of seeds and altered embryo development altered seed morphology and delayed fruit abscission.

- Immunolocalization analysis indicated a significant decrease of epitope-bound AGP proteins content in flower abscission zones of P4H3 RNAi lines compared to the wild type. However, no changes in AGP proteins content were observed in fruit abscission zones at the red ripe stage.

- These results indicate that alterations in the expression of P4Hs alter the content of substrate proteins such as AGPs which might be linked to flower abscission initiation and progression

WORK IN PROGRESS

- Screening of Tomato fruit ripening Yeast 2 Hybrids libraries with P4H3 as bait

- Stable tomato transformation of P4H3 cDNA with epitope tags (HA, FLAG) for pull down assays

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Collaborators

Mondher Bouzayen
Genomic and Biotechnology of Fruit,
INRA-INP-ENSAT, Toulouse, France

Kostas Billiaderis, Mourtzinos I., Lazaridou A.,
Food Science Dept
Aristotle University of Thessaloniki, Greece

Plant Phenomics Facility
University of Nottingham, UK

Reiuden Allen,
University of Oslo, Norway

Jean Claude Mollet
Azeddine Driouich
University of Rouen, France