



LEIBNIZ UNIVERSITÄT HANNOVER  
DEPARTMENT OF MOLECULAR PLANT BREEDING  
INSTITUTE OF PLANT GENETICS



**MOLECULAR STUDIES ON THE ANTHOCYANIN PATHWAYS  
IN RED- AND WHITE-FLOWERING POINSETTIAS  
(*Euphorbia pulcherrima* Willd. ex. Klotzsch)**

**M.Sc. Vinicius Vilperte**

**Supervisor**

**Dr. Thomas Debener**

**1 – FlowerPower Project**

**2 – Poinsettia and the *white paradox***

**3 – Transcriptome analysis**

**4 – Transient gene expression**

**5 – Follow up analysis**

**6 – References**

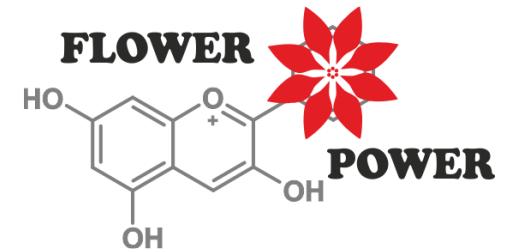
***Establishing a new generation of horticulturists: Multidisciplinary approach for breeding innovative novelties using classical and biotechnological methods***



Horizon 2020  
European Union Funding  
for Research & Innovation



MARIE CURIE ACTIONS



## European Industrial Doctorates (EID)

- Joint doctoral training → at least one academic partner entitled to award doctoral degrees, and at least one partner from outside academia, primarily enterprise.
- Each ESR is enrolled in a doctoral programme and supervised by supervisors from the academic and non-academic sector
- Aimed to develop skills inside and outside academia that respond to public and private sector needs.
- The organisations should be established in at least two different EU or associated countries.

# 1. FlowerPower Project

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**ESR1 (Benjamin Walliser – TUV/Selecta)** - Anthochlor biosynthesis and its application in biotechnological breeding for novel flower colour

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**ESR2 (Vinicius Vilperte – LUH/Selecta)** - Molecular studies on the anthocyanin pathway in red and white-flowering roses and poinsettias



universität  
wien

**ESR3 (Carmen Stefanini - TUM/Selecta)** - Isolation and identification of the “poinsettia metabolites” and elucidation of their role in anthocyanidin and flavanol biosynthesis in poinsettia and roses



UNIVERSITY OF HELSINKI



**ESR4 (Rares Lucaciu – UNIVIE/Vertis)** - Bioinformatic analysis of next-generation sequencing data obtained from poinsettia, dahlia, rose and scab infected apple tissues



**ESR5 (Daria Nitarska – TUV/Selecta)** - Genome editing approach for the creation of blue flowering poinsettia

**selecta**<sup>one</sup>  
*we love to grow*

**ESR6 (Martina Kolarek – TUM/BayOZ)** - Isolation and identification of the “poinsettia metabolites” and elucidation of their role in anthocyanidin and flavanol biosynthesis in apple tissues

**vertis** Biotechnologie AG  
Accelerating Gene Discovery

**Bay|O|Z**  
Bayerisches Obstzentrum

### *Euphorbia pulcherrima* Willd. ex Klotzsch

- Euphorbiaceae family
- Mexico as center of origin<sup>1</sup>
- Genus *Euphorbia* contains 24 species
- Cassava (*Manihot esculenta* Crantz) and the castor oil plant (*Ricinus communis* L.)
- *Jatropha curcas* L. → Complete genome
- *E. esula* L. and *E. fischeriana* → Transcriptome<sup>2,3</sup>
- White poinsettias → Induced artificially by radioactive irradiation

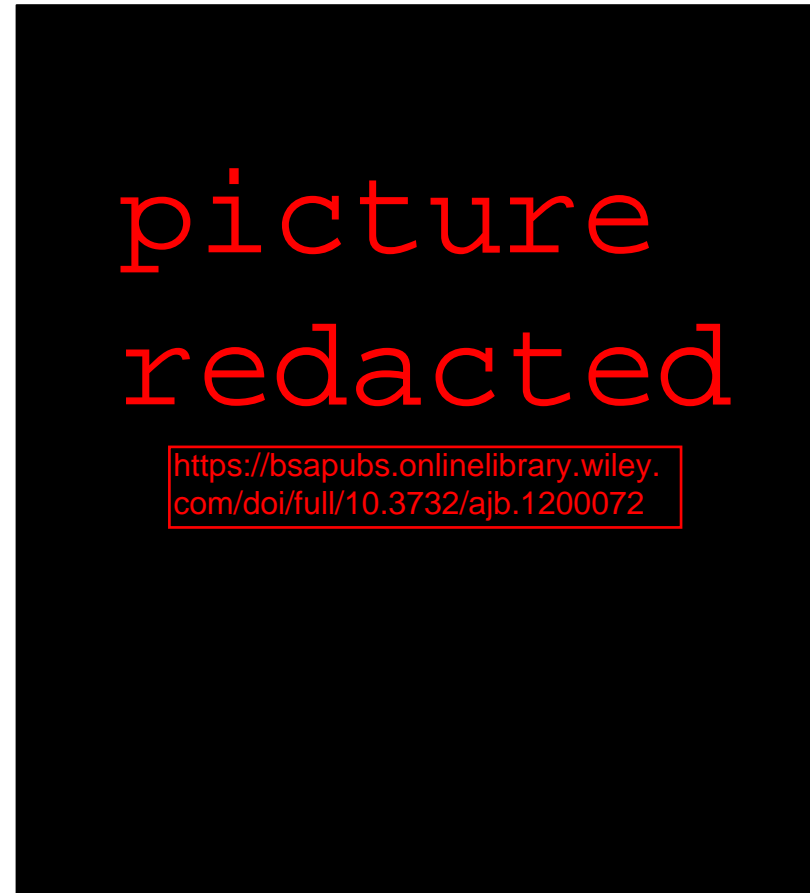


Figure: Trejo et al. 2012

Christmas  
Feelings  
Pearl



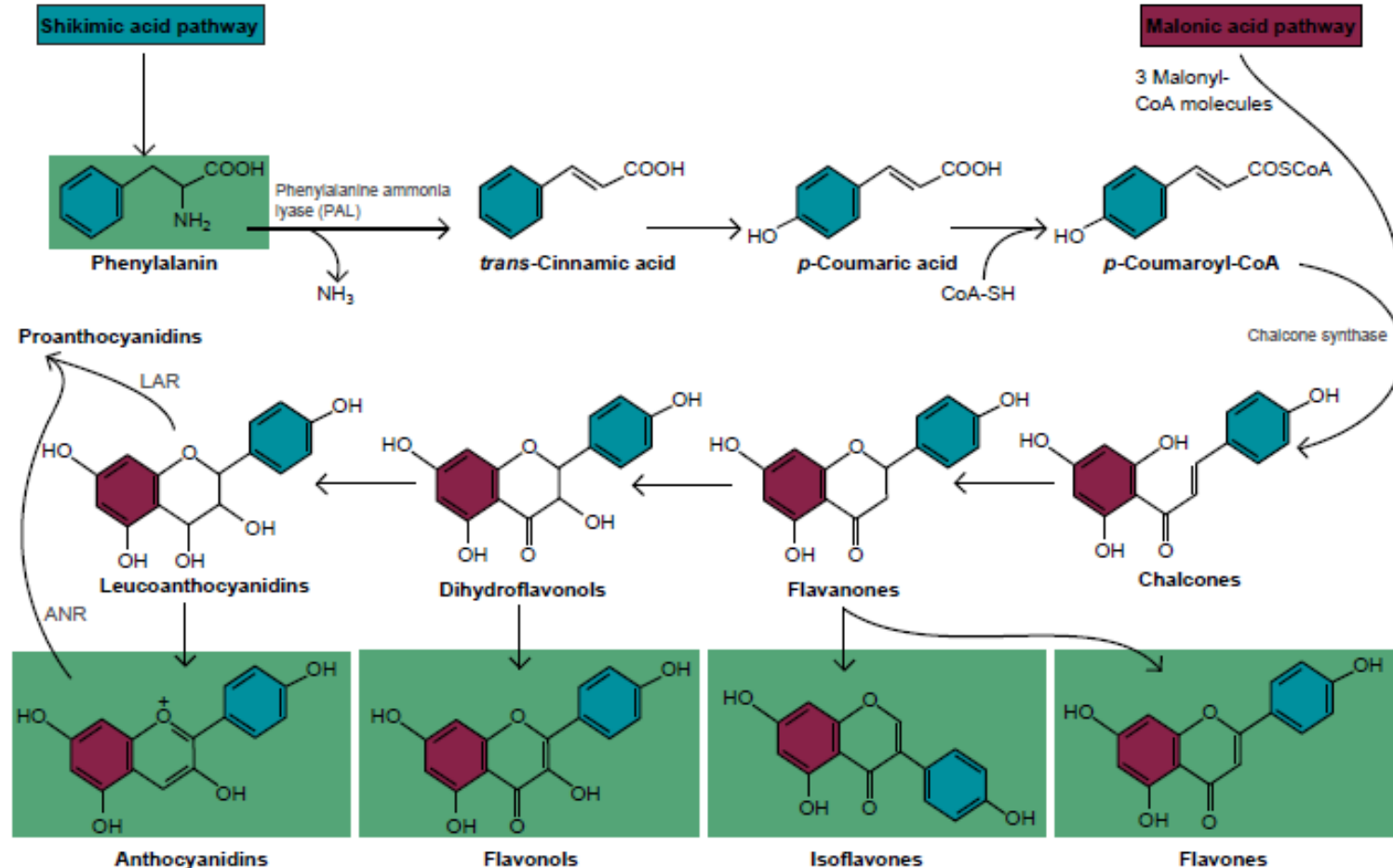
Christmas  
Feelings  
Wonder



Pictures: www.selecta-one.com

## White paradox

- Occurrence of uncoloured (=acyanic) varieties although gene expression and enzyme activities involved in the formation of red anthocyanin pigments can be determined.



# 2. Poinsettia and the *white paradox*

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Fig. 1

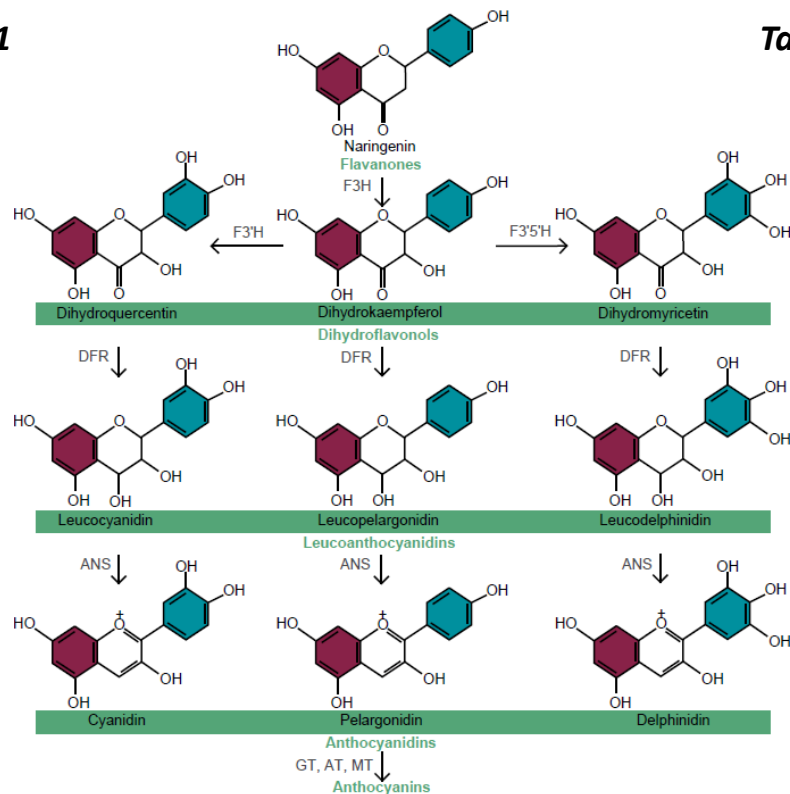


Table 2

Anthocyanin (mg kg <sup>-1</sup> FW)	Poinsettia cultivar		
	'Mars White'	'Mars Pink'	'Mira Red'
Cyanidin-3-galactoside	2.42 ± 0.80 a <sup>b</sup>	147.97 ± 8.13 a	316.26 ± 41.86 a
	7.24 ± 0.35 b	382.01 ± 36.53 c	1081.68 ± 19.74 c
	7.29 ± 0.68 b	300.95 ± 28.80 bc	1003.19 ± 38.88 bc
	6.71 ± 0.58 b	239.72 ± 24.65 b	959.53 ± 41.23 b
Cyanidin-3-glucoside	2.62 ± 0.12 a	566.91 ± 25.89 a	409.64 ± 50.19 a
	14.90 ± 0.81 b	1405.61 ± 136.07 c	2211.89 ± 48.91 b
	17.70 ± 1.33 b	1141.33 ± 89.25 bc	2043.63 ± 149.68 b
	14.61 ± 1.54 b	990.77 ± 90.70 b	2090.56 ± 104.81 b
Cyanidin-3-rutinoside	2.60 ± 0.78 a	94.13 ± 7.84 a	669.40 ± 65.00 a
	11.74 ± 0.85 b	431.60 ± 58.78 c	1228.72 ± 57.33 b
	11.74 ± 1.10 b	343.91 ± 39.86 bc	1209.90 ± 46.48 b
	11.07 ± 0.93 b	260.48 ± 22.23 b	1129.88 ± 44.09 b
Pelargonidin-3-glucoside	0.31 ± 0.16 a	43.85 ± 1.37 a	72.76 ± 13.80 a
	5.61 ± 0.35 b	273.40 ± 31.55 c	1485.81 ± 49.31 b
	4.56 ± 0.56 b	199.41 ± 15.02 b	1346.80 ± 137.20 b
	5.44 ± 0.67 b	170.82 ± 15.08 b	1398.77 ± 68.85 b
Pelargonidin-3-rutinoside	0.23 ± 0.07 a	8.09 ± 1.02 a	129.58 ± 23.78 a
	2.86 ± 0.09 b	53.26 ± 6.65 c	655.37 ± 44.01 b
	2.57 ± 0.29 b	41.78 ± 4.50 bc	666.82 ± 35.20 b
	2.73 ± 0.30 b	33.71 ± 3.46 b	622.83 ± 26.84 b



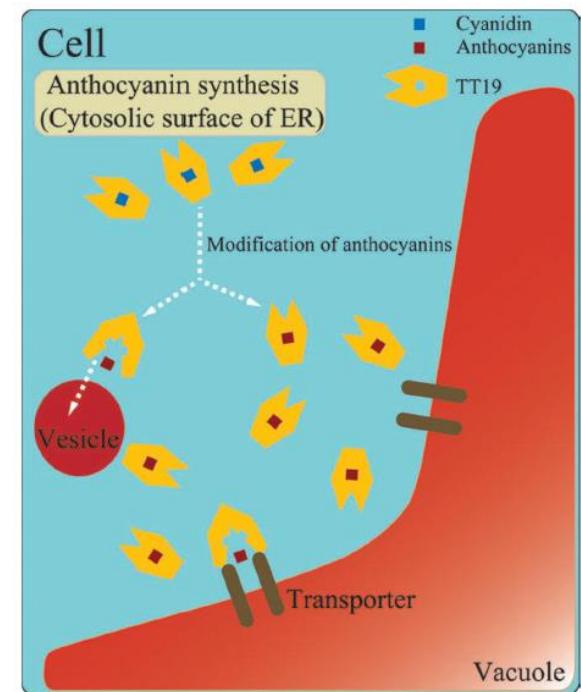
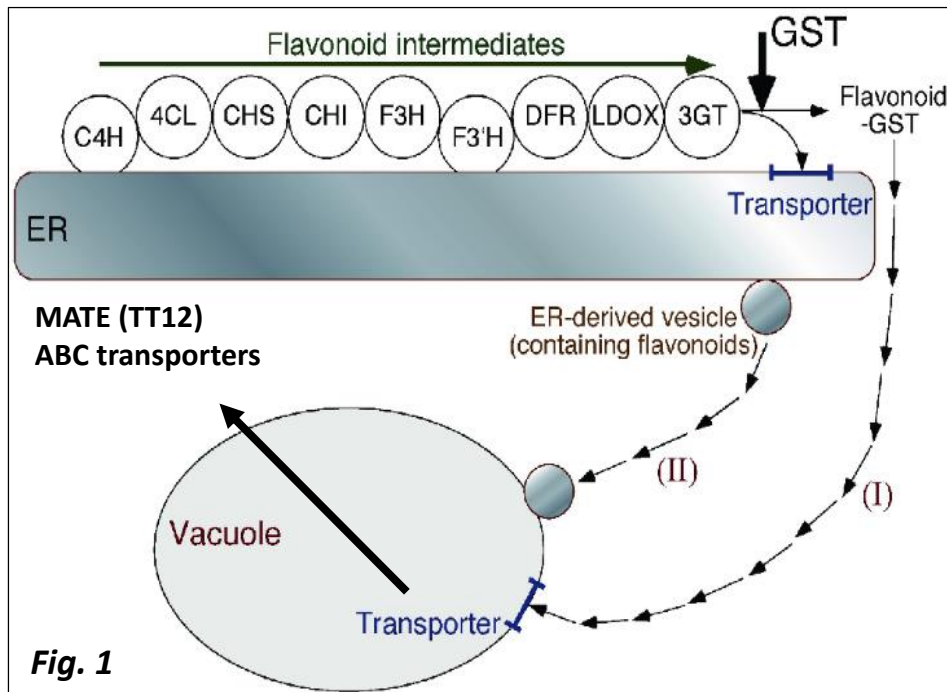
Table 1

Anthocyanin <sup>a</sup>	Standard used	Poinsettia cultivar			[M <sup>+</sup> ] (m/z)	MS <sup>2</sup> [M <sup>+</sup> ] (m/z)
		'Mars Pink'	'Mars White'	'Mira Red'		
cy-3-Galactoside	cy-3-Galactoside	x	x	x	449	287
cy-3-Glucoside	cy-3-Glucoside	x	x	x	449	287
cy-3-Rutinoside	cy-3-Rutinoside	x	x	x	595	449/287
pel-3-Glucoside	cy-3-Glucoside	x	x	x	433	271
pel-3-Rutinoside	cy-3-Rutinoside	x	x	x	579	433/271
cy-3-Rhamnoside	cy-3-Glucoside	x	x	x	433	287
cy-3-Xyloside	cy-3-Glucoside	x	x	x	419	287
cy-3-(6''Acetylglucoside)-5-glucoside	cy-3-Glucoside	x	x	x	653	287
cy-3-(6''Malonylglucoside)-5-glucoside	cy-3-Glucoside	x	x	x	697	535/449/287
pel-3-(6''Malonylglucoside)	cy-3-Glucoside	x	x	x	519	433/271
del-3-(2G-Xylosylrutinoside)	cy-3-Glucoside	x	x	x	743	597/435/303



### Anthocyanin transport

- Synthesized on the cytoplasmic surface of the endoplasmic reticulum (ER)
- Two models for anthocyanin import into vacuoles: I) transporter-mediated model and II) vesicle trafficking-mediated model<sup>1</sup>
- Vesicle-like structures (called anthocyanoplasts) and imported into the central vacuole probably in a vesicle fusion manner<sup>2</sup>





# 2. Poinsettia and the *white paradox*

## MYB-bHLH-WD40 protein complex

Fig. 1

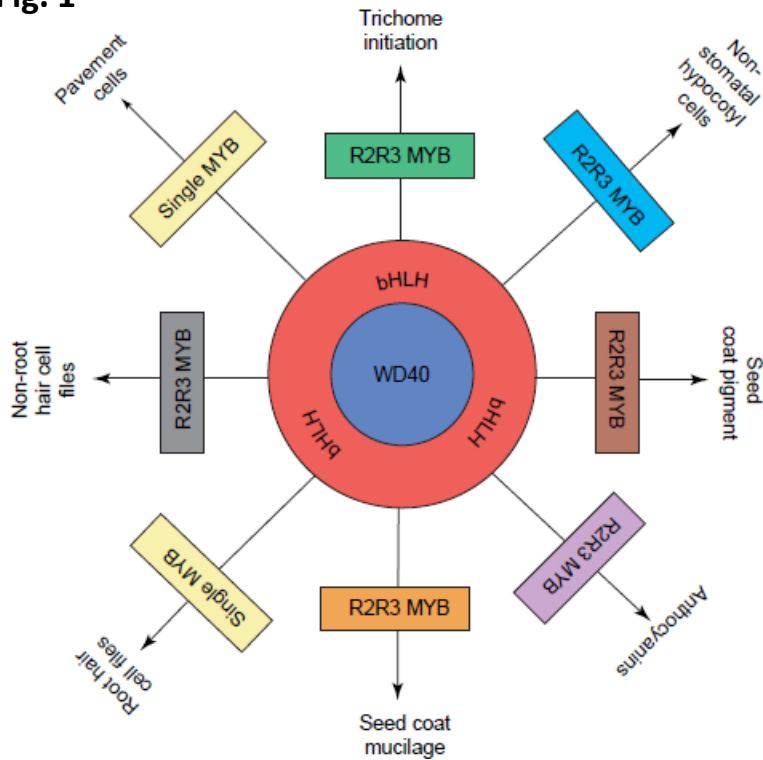


Fig. 2

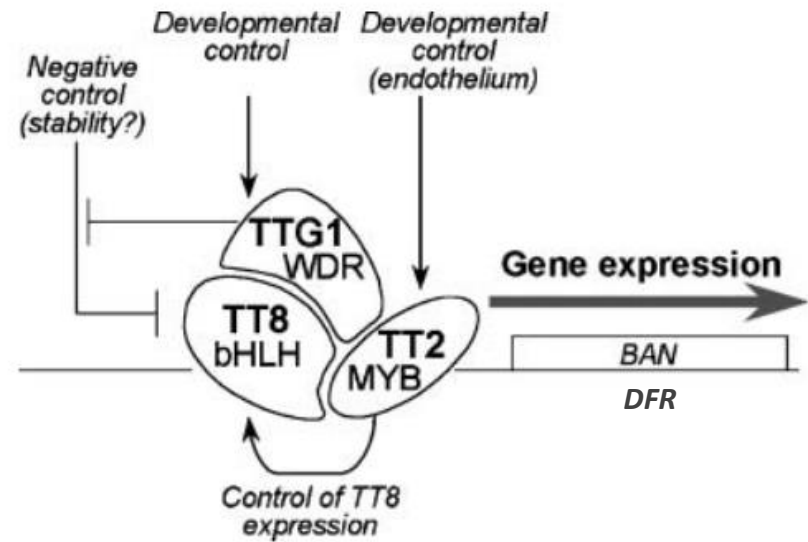
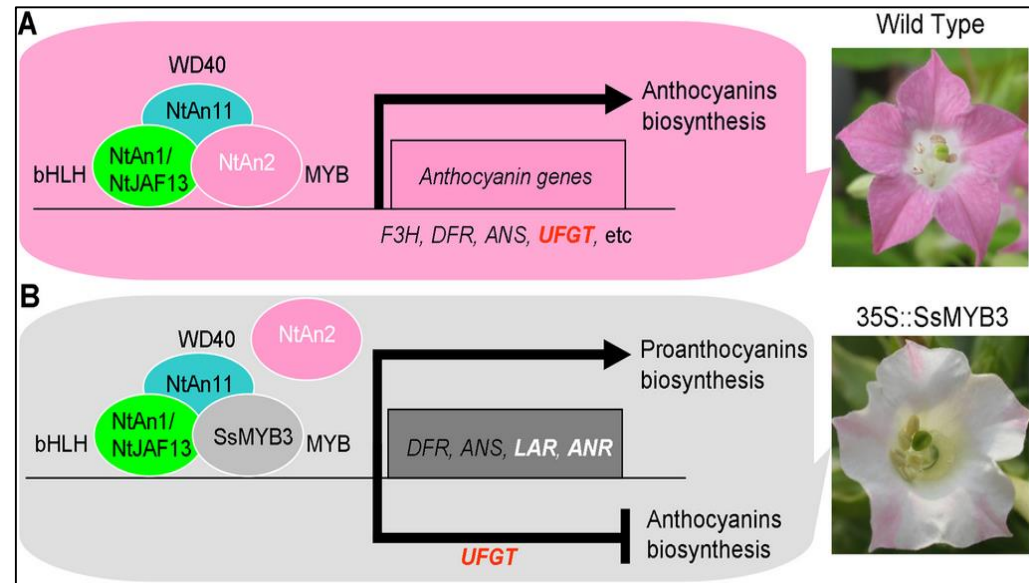


Fig. 3



# 3. Transcriptome analysis

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## Transcriptome assembly



Total RNA isolation  
Illumina cDNA library

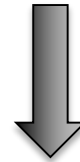


Illumina HiSeq2000  
~300M 2x100 *paired-end* reads



rRNA filtering  
Quality trimming

  
high quality ribosomal RNA databases  
Trimmomatic v0.32

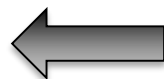


*de novo* Transcriptome  
assembly



Transcriptome  
draft

Transcriptome  
annotation



## Transcriptome assembly

### *Assembly stats*

Number of 'genes' → 55,191

Number of 'transcripts' → 166,153

Assembled bases → 230,721,719

N50 → 1956

### *Annotation stats*

83,440 contigs with annotation

13,840 unique proteins

Contig ID	UniProt ID	Coverage	E-value
TRINITY_DN11672_c0_g1_i1	SP1_ARATH	62,712	8,09E-16
TRINITY_DN11672_c0_g2_i1	SP1_ARATH	74,783	2,36E-156
TRINITY_DN11672_c0_g2_i2	SP1_ARATH	77,515	7,97E-124
TRINITY_DN11672_c0_g2_i3	SP1_ARATH	69,189	7,97E-124
TRINITY_DN11672_c0_g1_i3	SP1_ARATH	74,178	3,53E-112
TRINITY_DN11615_c0_g1_i1	SP1_ARATH	74,783	2,17E-156
TRINITY_DN11615_c0_g2_i1	SP1_ARATH	74,178	3,54E-112
TRINITY_DN11615_c0_g2_i2	SP1_ARATH	59,603	3,54E-112

Blast Coverage	Number of transcripts	Accumulated number of transcripts
100%	5937	5937
90%	1564	7501
80%	1049	8550
70%	919	9469
60%	877	10346
50%	834	11180
40%	877	12057
30%	805	12862
20%	722	13584
10%	256	13840

# 3. Transcriptome analysis

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## Differential Gene Expression (DEG)



Total RNA isolation  
Illumina cDNA library

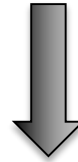


Illumina HiSeq2000  
2x100 paired-end reads

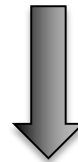


rRNA filtering  
Quality trimming

silva  
high quality ribosomal RNA databases  
Trimmomatic v0.32



Transcriptome mapping

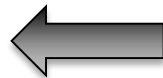


Read counting



RSEM  
x  
Kallisto

Differential Gene  
Expression (DEG)



DESeq2  
x  
edgeR

Plot of the  
results

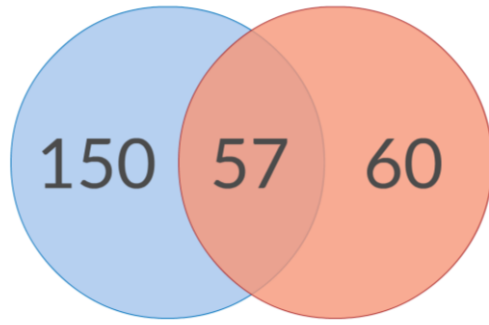
# 3. Transcriptome analysis

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## Kallisto

DESeq2

edgeR



## RSEM

DESeq2

edgeR

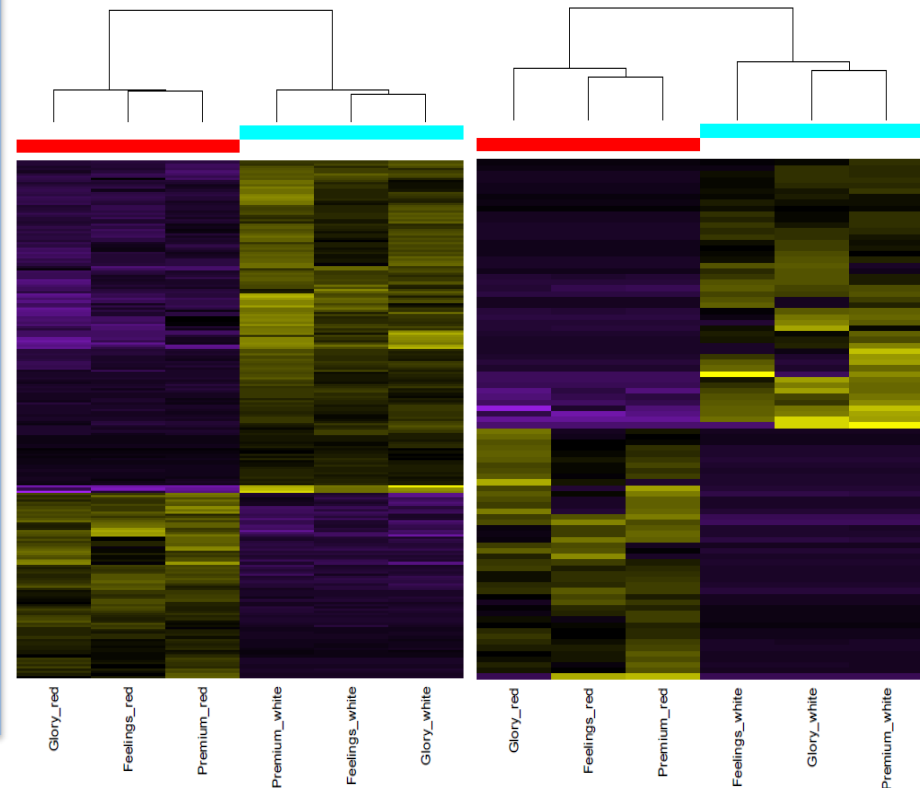
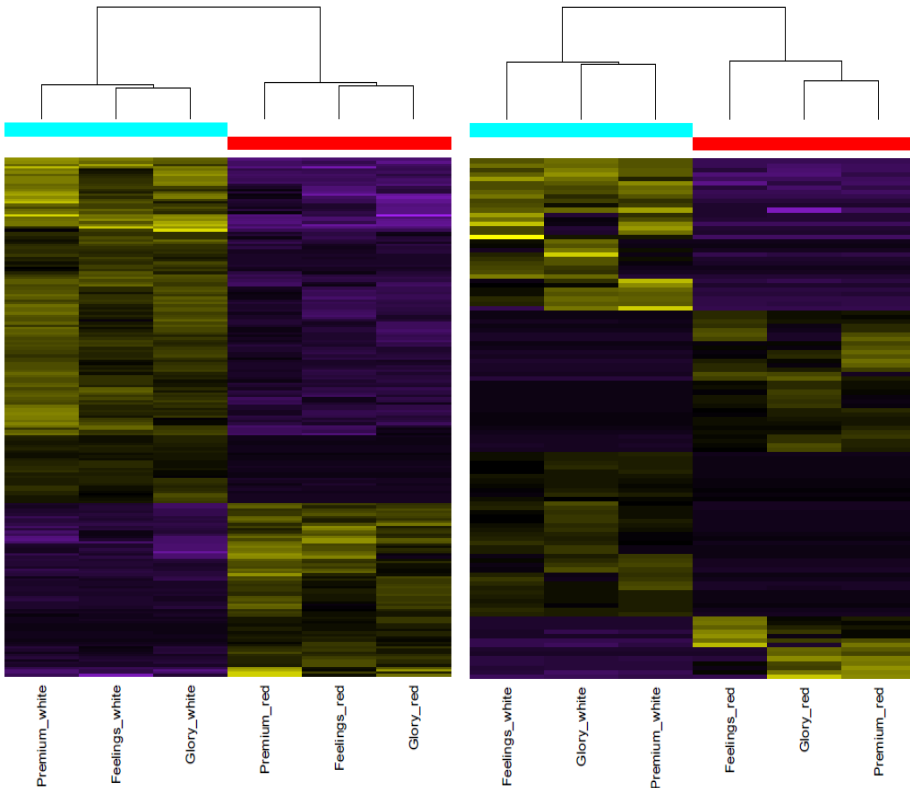
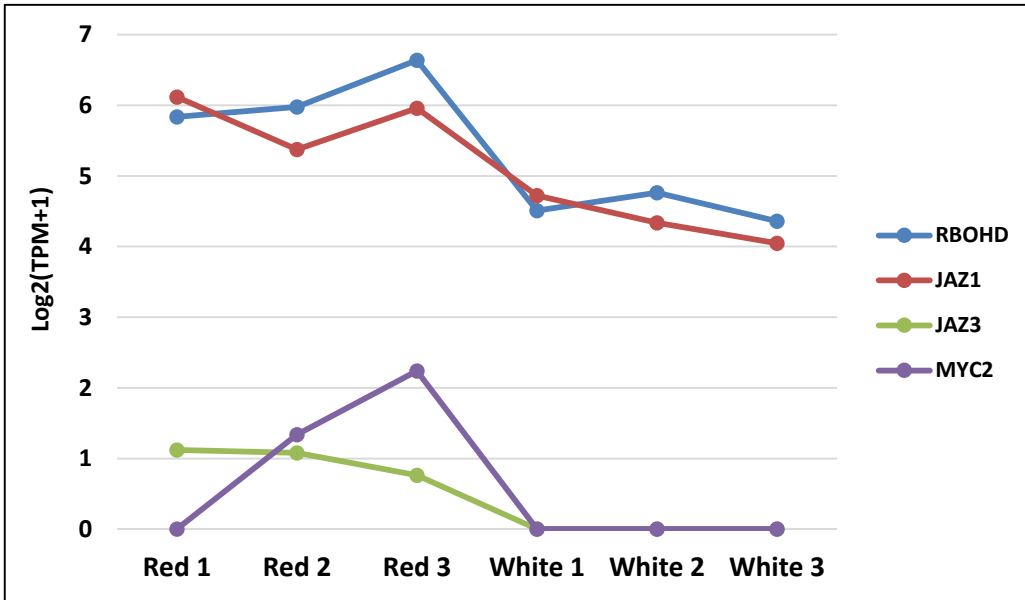


Fig.1: Ramsay and Glover (2005); Fig. 2: Baudry et al. (2004); Fig. 3: Zhu et al. (2015)

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UniProt ID	Protein description
RBOHD_ARATH	Respiratory burst oxidase homolog protein D
TI10A_ARATH	Protein TIFY 10A (Jasmonate ZIM domain-containing protein - JAZ1)
TIF6B_ARATH	Protein TIFY 6B (Jasmonate ZIM domain-containing protein - JAZ3)
MYC2_ARATH	Transcription factor MYC2 - bHLH transcription factor



The Plant Cell, Vol. 19: 2225–2245, July 2007, www.plantcell.org © 2007 American Society of Plant Biologists

## MYC2 Differentially Modulates Diverse Jasmonate-Dependent Functions in *Arabidopsis*<sup>TM</sup>

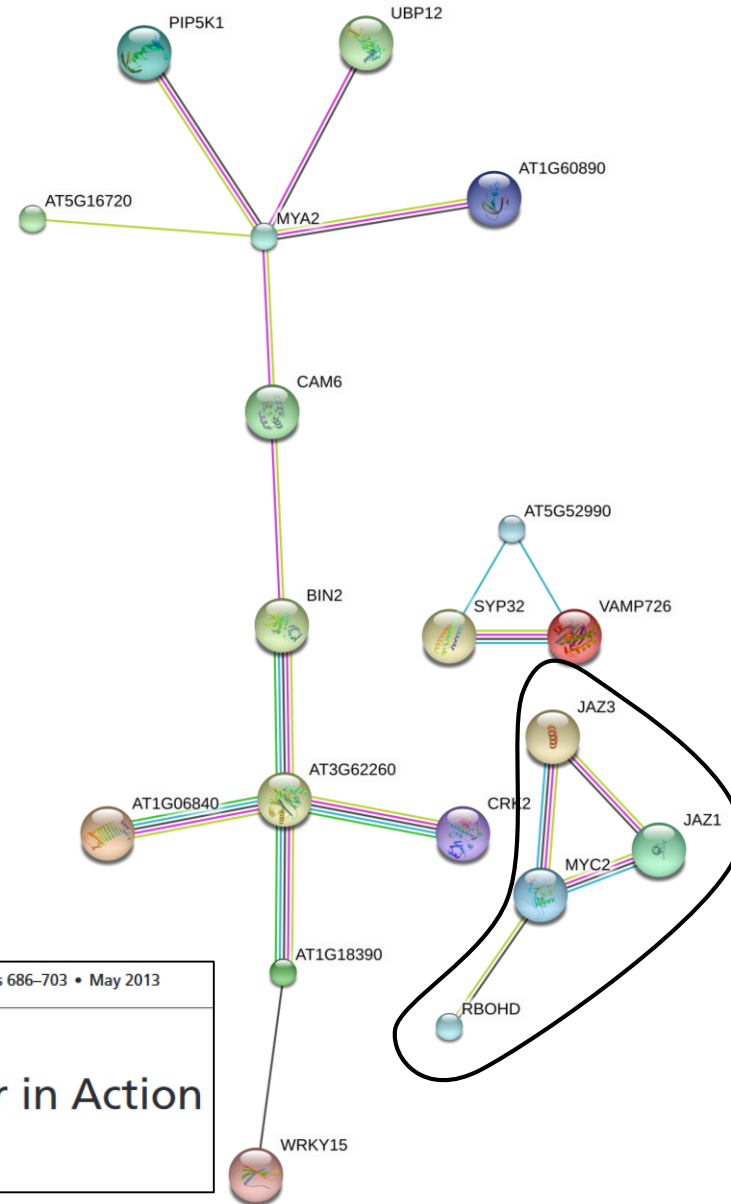
Bruno Dombrecht,<sup>a,1</sup> Gang Ping Xue,<sup>a</sup> Susan J. Sprague,<sup>b</sup> John A. Kirkegaard,<sup>b</sup> John J. Ross,<sup>c</sup> Gary P. Fitt,<sup>d</sup> Nasser Sewelam,<sup>a,e</sup> Peer M. Schenk,<sup>a</sup> John M. Manners,<sup>a</sup> and Kemal Kazan<sup>a,2</sup>

→ MYC2 is a positive regulator of flavonoid biosynthesis.

Molecular Plant • Volume 6 • Number 3 • Pages 686–703 • May 2013

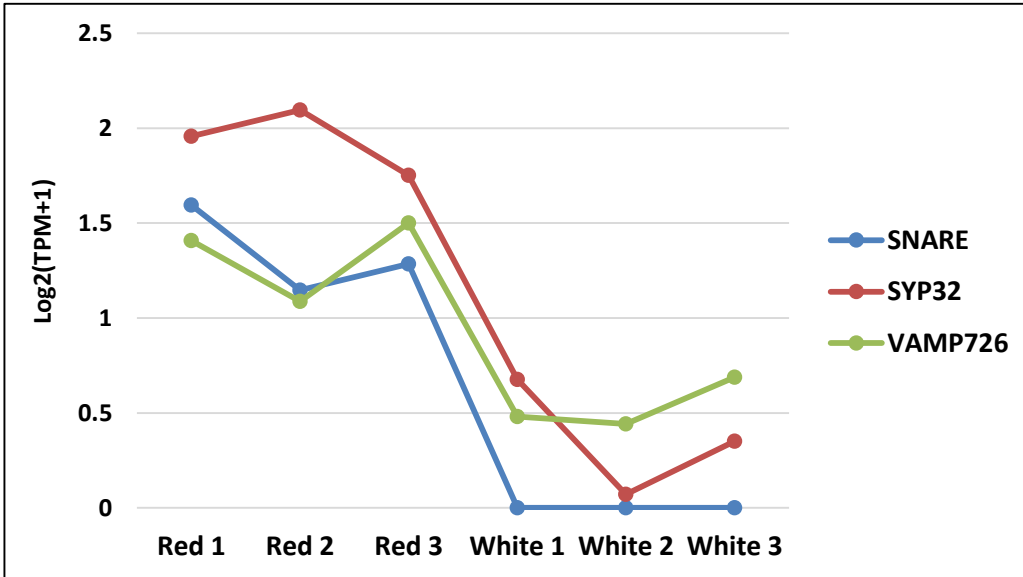
## MYC2: The Master in Action

Kemal Kazan<sup>1</sup> and John M. Manners



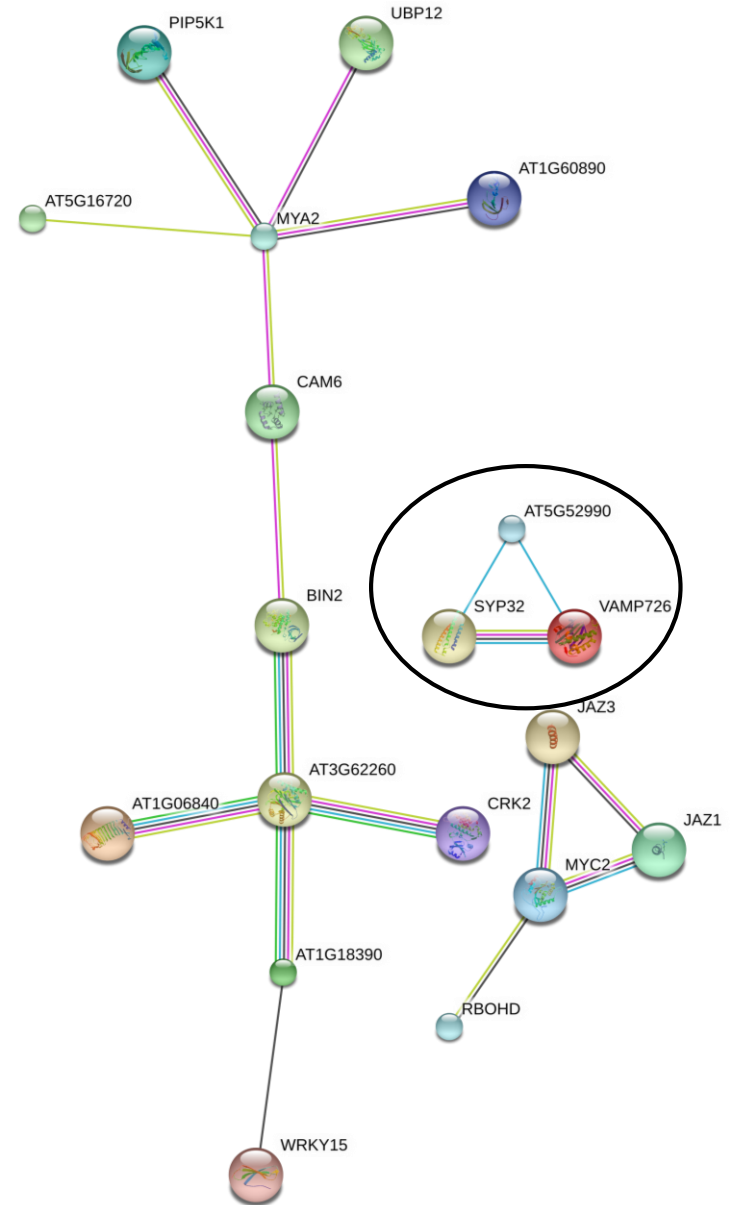
# 3. Transcriptome analysis

UniProt ID	Protein description
PHL22_ARATH	Phytolongin
SYP32_ARATH	Syntaxin-32
VA726_ARATH	Putative vesicle-associated membrane protein 726



Phytolongin → Non-SNARE longin protein involved in membrane-trafficking machinery

VAMP proteins → Involved in the targeting and/or fusion of transport vesicles to their target membrane

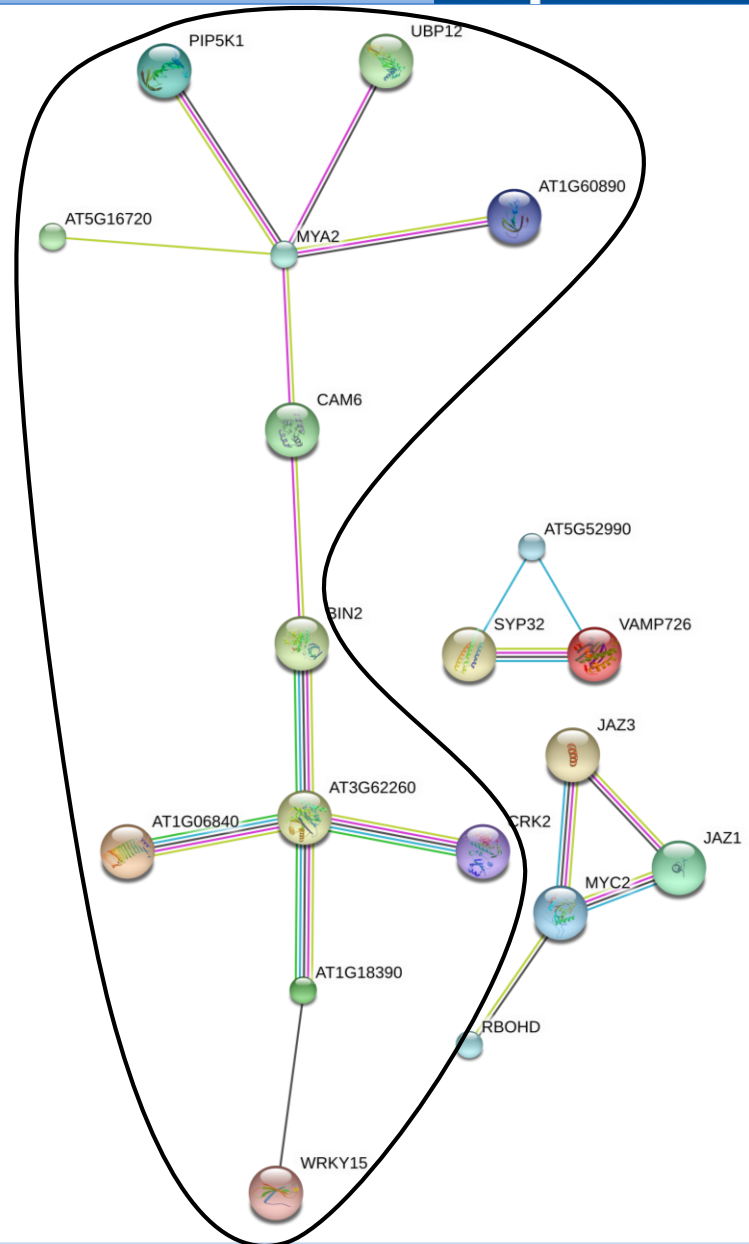
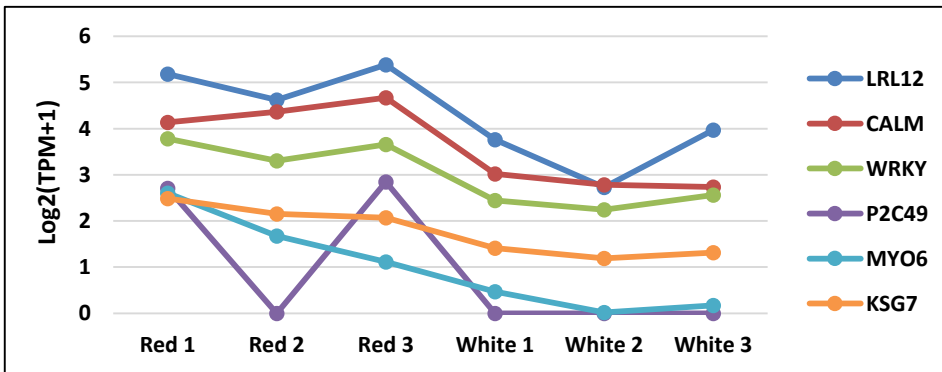
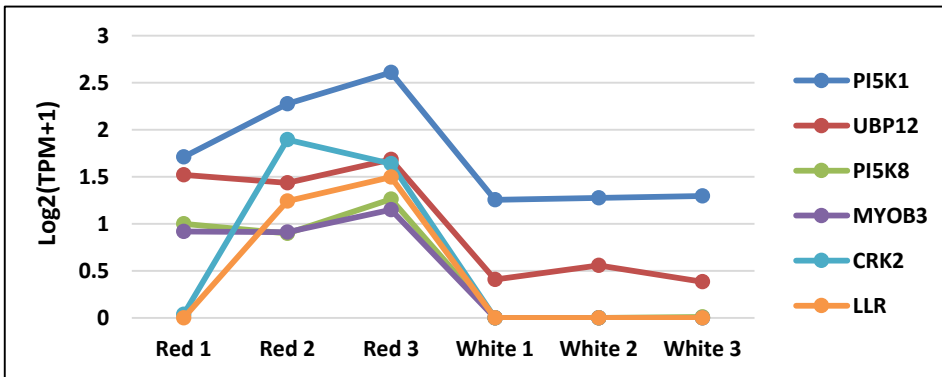




# 3. Transcriptome analysis

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UniProt ID	Protein description
PI5K1_ARATH	Phosphatidylinositol 4-phosphate 5-kinase 1
UBP12_ARATH	Ubiquitin carboxyl-terminal hydrolase 12
MYOB3_ARATH	Myosin-binding protein 3
P2C49_ARATH	Probable protein phosphatase 2C 49
CRK2_ARATH	Cysteine-rich receptor-like protein kinase 2
Y1684_ARATH	Probable LRR receptor-like serine/threonine-protein kinase
PI5K8_ARATH	Phosphatidylinositol 4-phosphate 5-kinase 8
MYO6_ARATH	Myosin-6
CALM_MEDSA	Calmodulin
WRK15_ARATH	WRKY transcription factor 15
LRL12_ARATH	Leaf rust 10 disease-resistance locus receptor-like protein kinase-like
KSG7_ARATH	Shaggy-related protein kinase eta

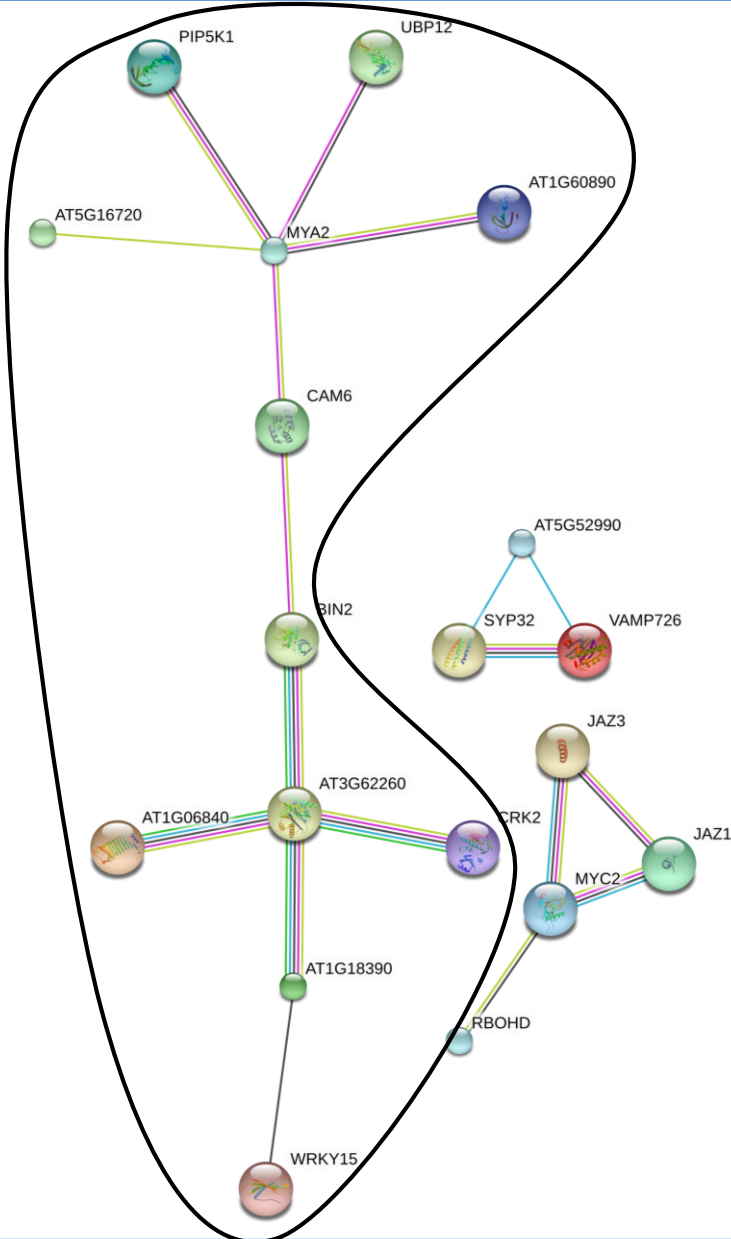
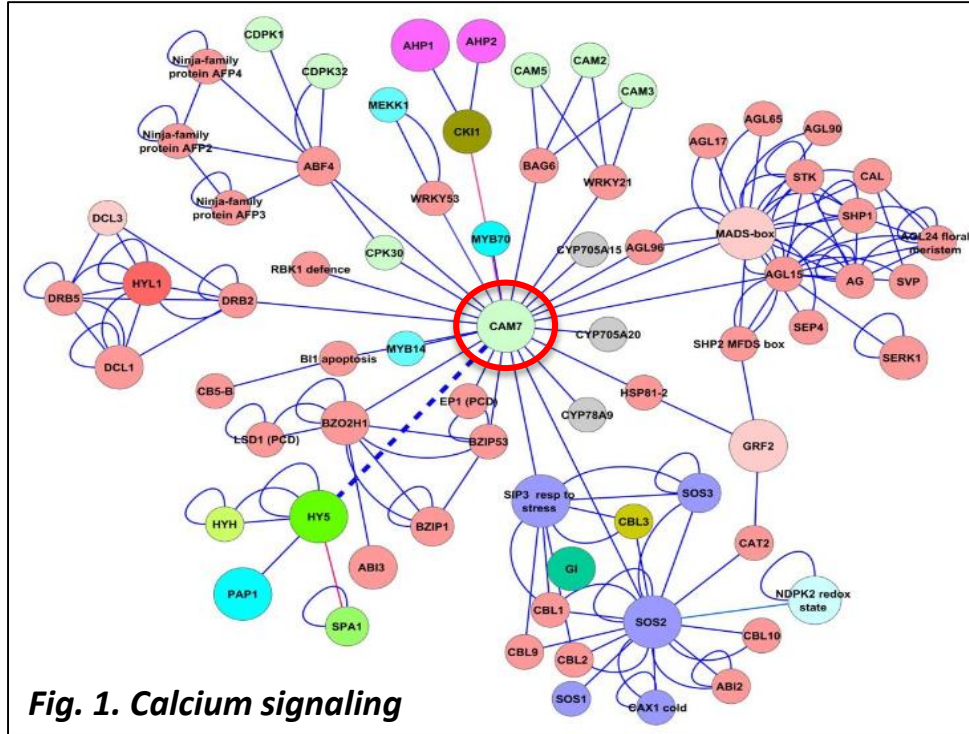


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→ Involvement of Ca<sup>2+</sup>, calmodulin, and protein kinases, in the induction of anthocyanin biosynthesis<sup>1</sup>



**FEBS**  
*Letters*

journal homepage: [www.FEBSLetters.org](http://www.FEBSLetters.org)



HY5 regulates anthocyanin biosynthesis by inducing the transcriptional activation of the MYB75/PAP1 transcription factor in *Arabidopsis*

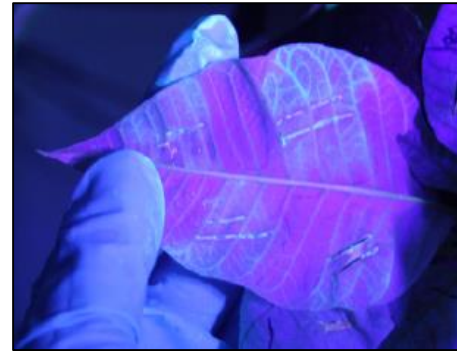


Dong Ho Shin <sup>a,1</sup>, MyungGoo Choi <sup>a,1</sup>, Keunhwa Kim <sup>b</sup>, Geul Bang <sup>a</sup>, Misuk Cho <sup>a</sup>, Sang-Bong Choi <sup>c</sup>, Giltsu Choi <sup>b</sup>, Youn-Il Park <sup>a,\*</sup>

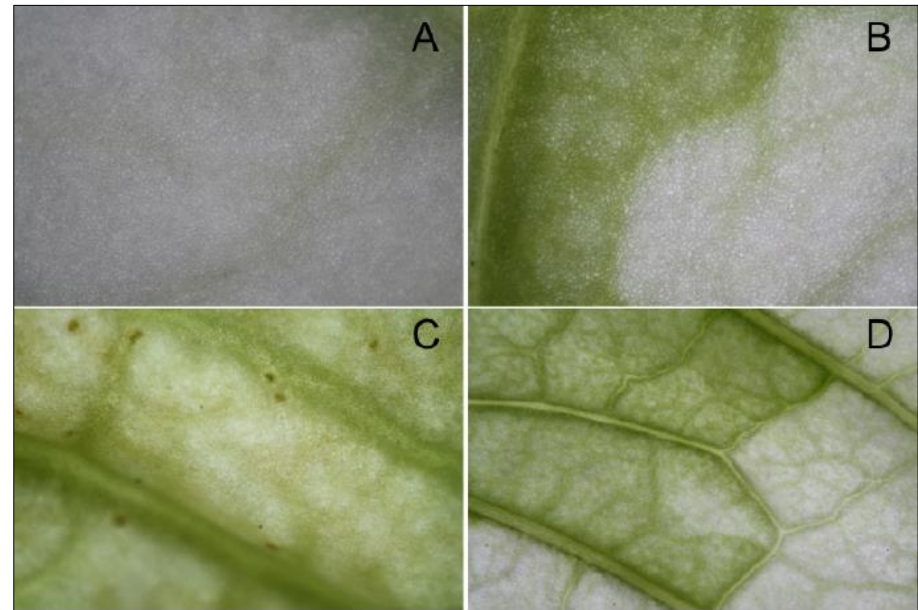
## Transient expression in Poinsettia

- Glutathione S-transferase (GST) infiltration

Plasmid/Strain	Plant genotype	Plant phenotype	Infiltration events	Events with visible transformation 3 dai.
Red bract GST/ EHA 105 or GV 105	3010	white	8	0
	3174	white	8	0
	3119	white	6	0
	56-1	sparkle	8	0
	3045	white	1	0
An9/ EHA 105	3010	white	3	0
	3174	white	8	0
	3119	white	8	0
	56-1	sparkle	8	0
	3122	red	1	0
An9/ GV3101	3010	white	4	0
	3174	white	9	0
	3119	white	9	0
	56-1	sparkle	9	0
	3122	red	1	0
Bz2/ EHA 105	3010	white	4	0
	3174	white	9	0
	3119	white	9	0
	56-1	sparkle	9	0
	3122	red	1	0
Bz2/ GV3101	3010	white	3	0
	3174	white	8	0
	3119	white	8	0
	56-1	sparkle	8	0
	3122	red	1	0
GFP '16e'	3010	white	8	7
	3174	white	12	11
	3119	white	10	9
	56-1	sparkle	12	0
	3045	white	1	1
	3122	red	1	0



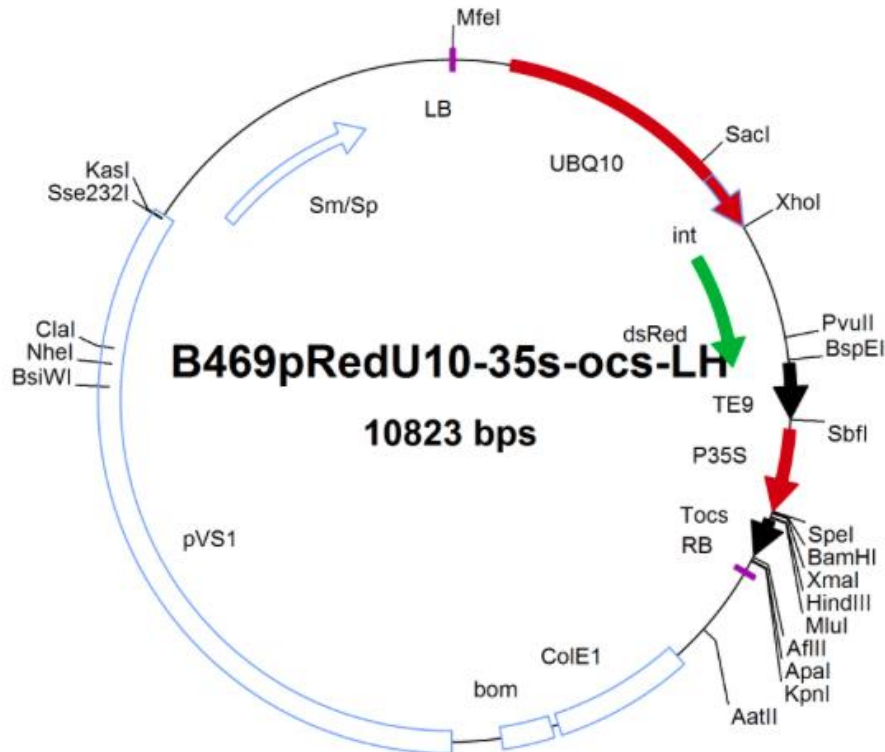
GFP '16e' infiltration in Poinsettia



A: Genotype 3174 bract not infiltrated. B and D: Genotype 3174 infiltrated with EHA 105 *Agrobacterium* strain, green coloration in the infiltration area. C: Genotype 3119 infiltrated with EHA 105 *Agrobacterium* strain, visible necrotic cells in infiltration area. 14 days after infiltration.

## Transient expression in Poinsettia

### UBQ10:dsRed



### *Lobelia erinus*



### *Calibrachoa*



### *Euphorbia pulcherrima*



dsRed cloned in several *Agrobacterium* strains

- GV3101
- GV2260
- EHA.105
- ABI



# 4. Transient gene expression

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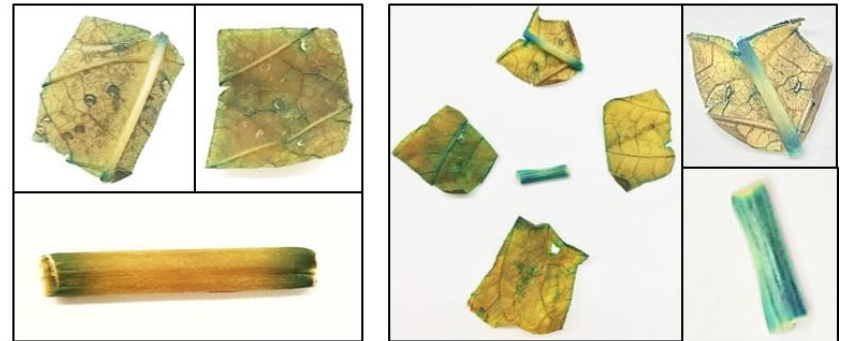
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## Transient expression in Poinsettia

### 35S:GUS

*Leaf sections incubated overnight with bacteria solution*

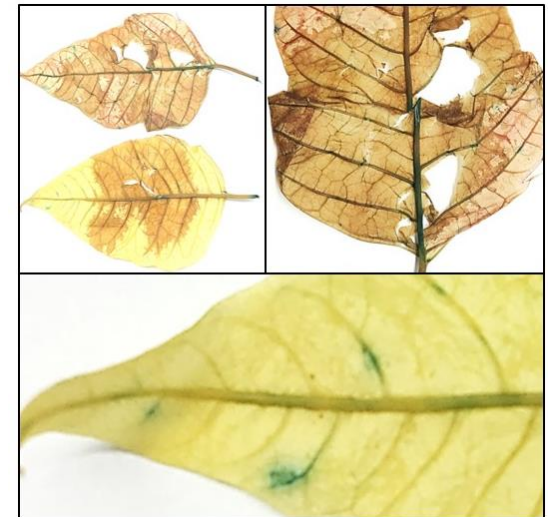
#### Positive control



#### Bracts kept on the plant



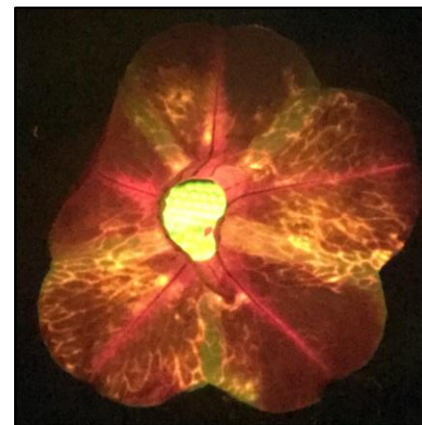
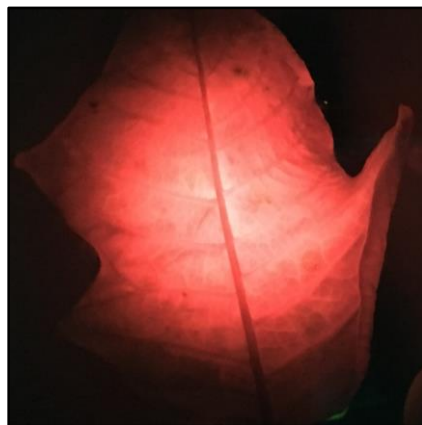
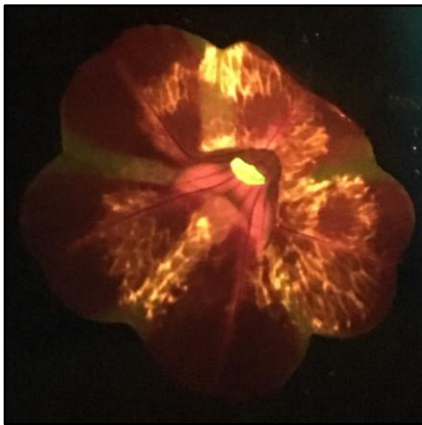
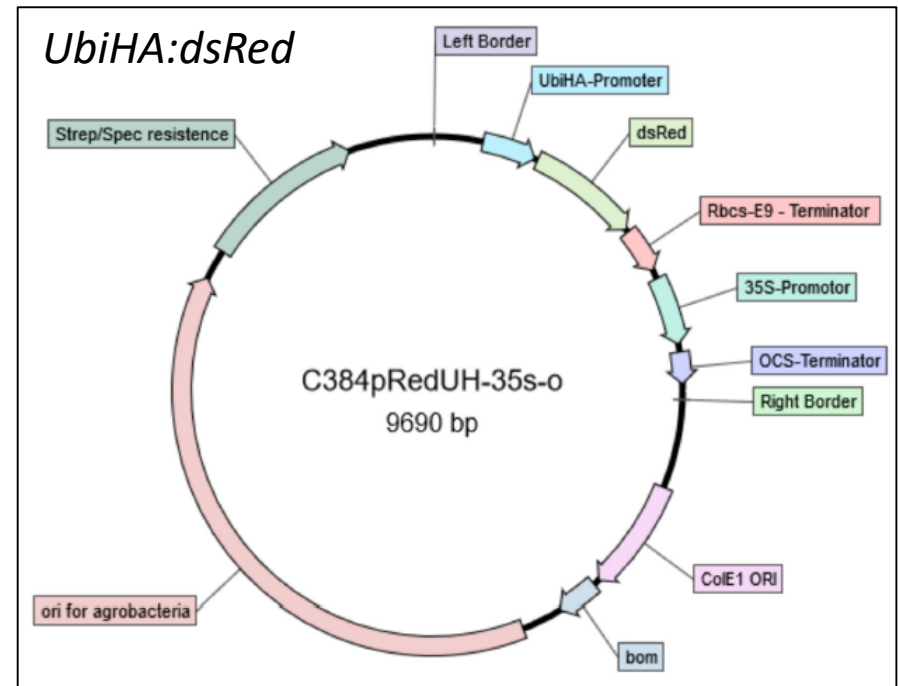
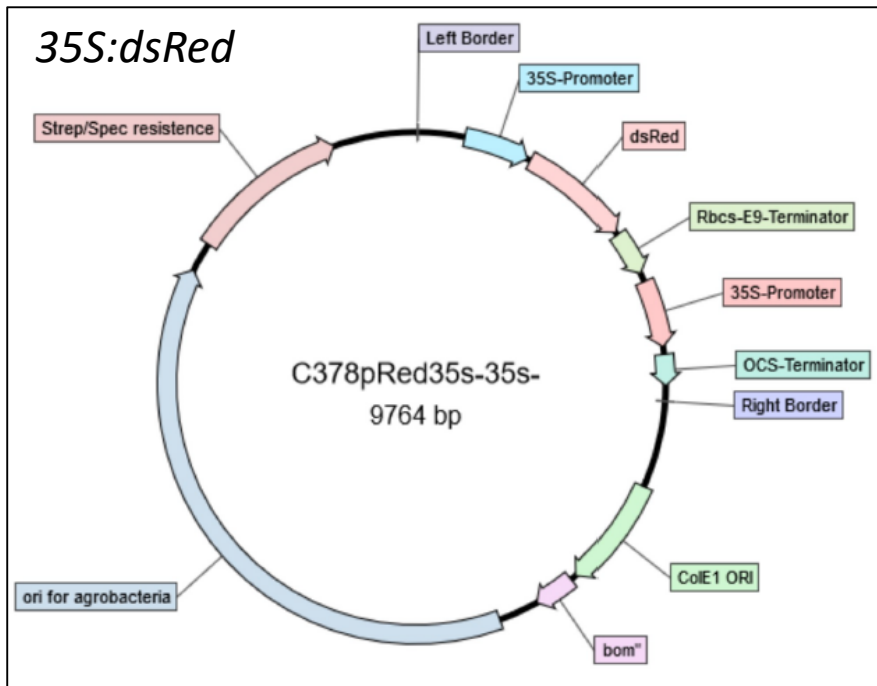
#### Bracts kept in a humid plastic box



# 4. Transient gene expression

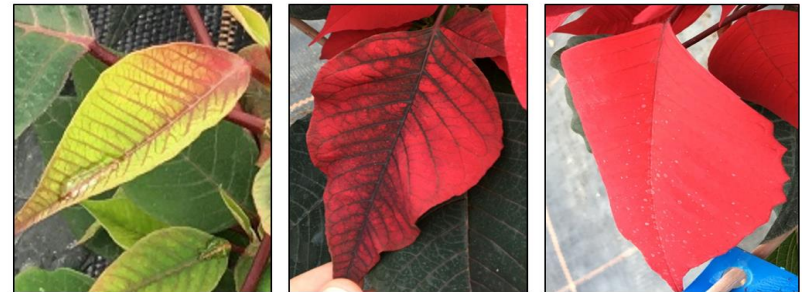
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## Transient expression in Poinsettia



## 5. Follow up analysis

- PacBio Single Molecule, Real-Time (SMRT®) will be performed from the same homozygous genotype
  - Iso-Seq method → generates full-length cDNA sequences (5' end of transcripts to the poly-A tail) eliminating the need for transcriptome reconstruction using isoform-inference algorithms;
  - Capable to capture full-length isoforms up to 10kb;
- New Illumina RNA-Seq for one red x white pair
  - 3 color development stages;
  - 3 biological replicates;
- Small RNA-Seq
  - Look for miRNAs and/or siRNAs.



STAGE 1

STAGE 2

STAGE 3



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