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
Content of the presentation

1 – FlowerPower Project

2 – Poinsettia and the *white paradox*

3 – Transcriptome analysis

4 – Transient gene expression

5 – Follow up analysis

6 – References
1. FlowerPower Project

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

**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

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

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

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

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 – TUW/Selecta) - Genome editing approach for the creation of blue flowering poinsettia

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
Euphorbia pulcherrima Willd. ex Klotzsch

- Euphorbiaceae family
- Mexico as center of origin\(^1\)
- 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\(^2,3\)
- White poinsettias → Induced artificially by radioactive irradiation

\(^1\)Trejo et al. 2012; \(^2\)Horvath et al. 2008; \(^3\)Barrero et al. 2011.
2. Poinsettia and the **white paradox**

**White paradox**

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

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[Figure: Taiz and Zeiger (2010), Grotewold (2006), modified by M.Sc. Dóra Klára Pinczinger]
2. Poinsettia and the white paradox

Fig. 1: Tanaka et al. (2008), modified by M.Sc. Dóra Klára Pinczinger; Tables 1 and 2: Slatnar et al. 2013
2. Poinsettia and the white paradox

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\(^1\)

- Vesicle-like structures (called anthocyanoplasts) and imported into the central vacuole probably in a vesicle fusion manner\(^2\)

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\(^1\)Grotewold (2006); \(^2\)Gomez et al. (2011); Fig.1: Grotewold (2006), modified; Fig. 2: Sun et al. (2012)
2. Poinsettia and the white paradox

MYB–bHLH–WD40 protein complex

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

Transcriptome assembly

Total RNA isolation
Illumina cDNA library

Illumina HiSeq2000
~300M 2x100 paired-end reads

rRNA filtering
Quality trimming

Trimmomatic v0.32

silva

de novo Transcriptome assembly

Transcriptome draft

Transcriptome annotation

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

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

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

Differential Gene Expression (DEG)

Total RNA isolation
Illumina cDNA library

Illumina HiSeq2000
2x100 paired-end reads

rRNA filtering
Quality trimming

Trimmomatic v0.32

Trancriptome mapping

Read counting

DESeq2
edgeR

Differential Gene Expression (DEG)

RSEM
Kallisto

Plot of the results

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

Kallisto

DESeq2  edgeR

150  57  60

RSEM

DESeq2  edgeR

175  48  43

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

<table>
<thead>
<tr>
<th>UniProt ID</th>
<th>Protein description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBOHD_ARATH</td>
<td>Respiratory burst oxidase homolog protein D</td>
</tr>
<tr>
<td>T110A_ARATH</td>
<td>Protein TIFY 10A (Jasmonate ZIM domain-containing protein - JAZ1)</td>
</tr>
<tr>
<td>TIF6B_ARATH</td>
<td>Protein TIFY 6B (Jasmonate ZIM domain-containing protein - JAZ3)</td>
</tr>
<tr>
<td>MYC2_ARATH</td>
<td>Transcription factor MYC2 - bHLH transcription factor</td>
</tr>
</tbody>
</table>

\[ \text{MYC2 Differentially Modulates Diverse Jasmonate-Dependent Functions in Arabidopsis™} \]

\[ \text{MYC2: The Master in Action} \]

\[ \rightarrow \text{MYC2 is a positive regulator of flavonoid biosynthesis.} \]
3. Transcriptome analysis

<table>
<thead>
<tr>
<th>UniProt ID</th>
<th>Protein description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHL22_ARATH</td>
<td>Phytolongin</td>
</tr>
<tr>
<td>SYP32_ARATH</td>
<td>Syntaxin-32</td>
</tr>
<tr>
<td>VA726_ARATH</td>
<td>Putative vesicle-associated membrane protein 726</td>
</tr>
</tbody>
</table>

**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

<table>
<thead>
<tr>
<th>UniProt ID</th>
<th>Protein description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PISK1_ARATH</td>
<td>Phosphatidylinositol 4-phosphate 5-kinase 1</td>
</tr>
<tr>
<td>UBP12_ARATH</td>
<td>Ubiquitin carboxyl-terminal hydrolase 12</td>
</tr>
<tr>
<td>MYOB3_ARATH</td>
<td>Myosin-binding protein 3</td>
</tr>
<tr>
<td>P2C49_ARATH</td>
<td>Probable protein phosphatase 2C 49</td>
</tr>
<tr>
<td>CRK2_ARATH</td>
<td>Cysteine-rich receptor-like protein kinase 2</td>
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<tr>
<td>Y1664_ARATH</td>
<td>Probable LRR receptor-like serine/threonine-protein kinase</td>
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<td>PISK8_ARATH</td>
<td>Phosphatidylinositol 4-phosphate 5-kinase 8</td>
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<td>MYO6_ARATH</td>
<td>Myosin-6</td>
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<tr>
<td>CALM_MEDSA</td>
<td>Calmodulin</td>
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<tr>
<td>WRK15_ARATH</td>
<td>WRKY transcription factor 15</td>
</tr>
<tr>
<td>LRL12_ARATH</td>
<td>Leaf rust 10 disease-resistance locus receptor-like protein kinase-like</td>
</tr>
<tr>
<td>KSG7_ARATH</td>
<td>Shaggy-related protein kinase eta</td>
</tr>
</tbody>
</table>

**Log2(TPM+1)**

![Graph showing Log2(TPM+1) for different conditions](image1)

![Graph showing Log2(TPM+1) for different conditions](image2)
3. Transcriptome analysis

→ Involvement of Ca2+, calmodulin, and protein kinases, in the induction of anthocyanin biosynthesis.

Fig. 1. Calcium signaling

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

Dong Ho Shin, MyungGoo Choi, Keunhwa Kim, Geul Bang, Misuk Cho, Sang-Bong Choi, Giltsu Choi, Youn-Il Park.

Vitrac et al. 2000; Fig.1: Bulgakov et al. 2016.
4. Transient gene expression

Transient expression in Poinsettia

- Glutathione S-transferase (GST) infiltration

<table>
<thead>
<tr>
<th>Plasmid/Strain</th>
<th>Plant genotype</th>
<th>Plant phenotype</th>
<th>Infiltration events</th>
<th>Events with visible transformation 3 dai.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red bract GST/ EHA105 or GV105</td>
<td>3010 3174 3119 56-1 3045</td>
<td>white white white sparkle white</td>
<td>8 8 6 8 1</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>An9/ EHA105</td>
<td>3010 3174 3119 56-1 3122</td>
<td>white white white sparkle red</td>
<td>3 8 8 8 1</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>An9/ GV3101</td>
<td>3010 3174 3119 56-1 3122</td>
<td>white white white sparkle red</td>
<td>4 9 9 9 1</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>Bz2/ EHA105</td>
<td>3010 3174 3119 56-1 3122</td>
<td>white white white sparkle red</td>
<td>3 9 9 9 1</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>Bz2/ GV3101</td>
<td>3010 3174 3119 56-1 3122</td>
<td>white white white sparkle red</td>
<td>8 8 8 8 1</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>GFP ‘16e’</td>
<td>3010 3174 3119 56-1 3045 3122</td>
<td>white white white white red</td>
<td>8 12 10 12 1 1</td>
<td>7 11 9 0 0 0</td>
</tr>
</tbody>
</table>

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.

Figures: M.Sc. Dóra Klára Pinczinger
4. Transient gene expression

Transient expression in Poinsettia

**UBQ10:dsRed**

*dsRed* cloned in several *Agrobacterium* strains
- GV3101
- GV2260
- EHA.105
- ABI

*Lobelia erinus*

*Calibrachoa*

*Euphorbia pulcherrima*
4. Transient gene expression

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

Transient expression in Poinsettia

**35S:dsRed**

![Diagram of 35S:dsRed expression vector](image1)

**UbiHA:dsRed**

![Diagram of UbiHA:dsRed expression vector](image2)

**Images**

![Flower expressing 35S:dsRed](image3)

![Flower expressing UbiHA:dsRed](image4)
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.


