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Mechanisms of complex programmed patterns of anthocyanin pigment formation in *Antirrhinum majus*

A thesis presented in partial fulfilment of the requirements for the degree of

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Abstract

*Antirrhinum majus* is a model plant used in flower pigmentation studies. Anthocyanin pigment production is mainly controlled by regulation of transcription of the anthocyanin biosynthetic genes. Two types of transcription factors, MYB and bHLH, together with a WD40 type co-regulator have been shown to regulate the transcription of the anthocyanin biosynthetic genes. In antirrhinum, in addition to the wild type Rosea1 phenotype, in which pigmentation occurs throughout the inner and outer epidermis of the petal, other complex pigmentation patterns are observed, such as anthocyanins being produced only in the outer (abaxial) epidermis of both lobes and upper tube region of the dorsal petals (rosea^dorsea^ phenotype). The major objective of this research project was to understand the genetic regulatory system leading to the development of the two different floral pigmentation patterns in antirrhinum as a means to understanding differential regulation of gene expression in similar cells.

Promoter deletion analysis coupled with linker scanning mutagenesis identified the -162 bp to -120 bp region of the *Rosea1* promoter as important for the regulation of the *Rosea1* gene. Four putative transcription factor-binding sites within this region: a W-box, a pyrimidine box, a DOF and a WRKY transcription factor binding site were shown to be important for *Rosea1* gene regulation.

Promoter deletion analysis carried out on the *rosea1^dorsea^* promoter showed that the proximal 187 bp deletion was, surprisingly, not responsible for the *rosea1^dorsea^* phenotype. Cloning and characterisation of the *Rosea1* promoter sequence from various *Antirrhinum* species and accessions verified this finding. The *rosea1^dorsea^* promoter analysis also indicated that -151 bp of the promoter was sufficient for its expression as well as for the maintenance of petal specific expression. The *rosea1^dorsea^* allele was also shown to encode a functional protein.

*In situ* hybridisation analysis showed that *Rosea1* transcripts were present in the inner and outer epidermis of the petal tissue of both wild type and *rosea1^dorsea^* petal tissue.
Vascular expression of the *Rosa1* mRNA is indicative of regulation of this gene through sugar or hormonal cues. However, *roseal* 

\[ \text{roseal}^{dorsea} \]

transcript levels (in *rosea^{dorsea}* ) were much lower than *Rosa1* (wild type). Lowered expression of *roseal* 

\[ \text{roseal}^{dorsea} \]

transcripts may be responsible for the overall weak pigmentation in the *rosea^{dorsea}* flowers. Analysis of the intron sequences of the two alleles revealed that many sequence changes were present in the intron 2 of *roseal* 

\[ \text{roseal}^{dorsea} \]

. These changes may lead to instability or the lower expression of the *roseal* 

\[ \text{roseal}^{dorsea} \]

mRNA and may be responsible for the *rosea^{dorsea}* phenotype. Another possibility is that a fourth *Myb* gene may be responsible for the *rosea^{dorsea}* phenotype.

The role of the *Deficiens* gene in direct regulation of *Roseal* was analysed by RNAi and bioinformatics-based methods. The presence of potential MADS box binding sites in the intron 2 region of the *Rosa1* allele indicated that *Rosa1* might be directly regulated by *Deficiens*. Initial experiments using transient assays did not support this suggestion. However, silencing of *Deficiens* in wild type antirrhinum buds led to the loss of anthocyanin pigments in the petals. Further analysis of the RNAi tissue using SEM revealed that the proper development of conical shaped epidermal cells was also affected. The RNAi tissue also developed chlorophyll pigments underscoring the plasticity of petal identity. This work demonstrated that proper expression of *Deficiens* is required throughout flowering for anthocyanin pigment production as well as maintenance of petal cell identity.

The current investigation revealed that the higher order regulation of the *Roseal* alleles in antirrhinum petals is much more complex than initially postulated.
Dedicated to the loving memory
of Chumpa aunty and Podisudu mami
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Abbreviations

IIA   TFIIA
2-ME  2-mercaptoethanol
3-AT  3-amino 1,2,4-triazole
ANS   Anthocyanidin synthase
BAP   Benzyl Amino Purine
Ω     Omega
bp    base pair
CHI   Chalcone isomerase
CHS   Chalcone synthase
CTAB  Cetyl Trimethyl Ammonium Bromide
Ci    Curie
DNA   DeoxyriboNucleic Acid
dNTP  deoxy Nucleotide TriPhosphate
DMF   Dimethylformamide
DMSO  Dimethyl sulfoxide
DFR   Dihydroflavonol 4-reductase
DTT   Dithiothreitol
EDTA  Ethylenediaminetetra-acetate
F3H   Flavanone 3-β-hydroxylase
F3'H  Flavonoid 3'-hydroxylase
F3'S'H Flavanoid 3'S'-hydroxylase
g     gram
GFP   Green Fluorescent Protein
GST   Glutathione-S-transferase
GMO   Genetically Modified Organism
Hepes 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
h     hour
IAA   Isoamylalcohol
IPTG  Isopropyl β-D-1-thiogalactopyranoside
kPa  kilo Pascal
L   litre
LB  L-Broth
LS  Linsmaier and Skoog
MES 2-(N-morpholino)ethanesulfonic acid
min  minutes
ms  miliseconds
N₂  nitrogen
mg  milligram
mL  millilitre
MS  Murshige and Skoog
MOPS 3-(N-morpholino)propanesulfonic acid
NAA  Naphthaleneacetic acid
OCS  Octopine synthase
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
PIPES  Piperazine-NN’-bis-2-ethanesulphonic acid
PMSF  Phenylmethylsulphonyl fluoride
Poly A  Polyadenylic Acid
PVP  Polyvinylpyrrolidine
rpm  revolutions per minute
3RT  UDP-rhamnose: anthocyanidin-3-O-glucoside rhamnosyltransferase
sec  seconds
SD  Synthetic Dropout
SDS  Sodium Dodecyl Sulphate
SEM  Sucrose-EDTA-Morpholinepropanesulfonic acid
SOT  Solenoid Opening Time
TAE  Tris-Acetate-EDTA
TAFs  TBP associated factors
TB  Terrfic Broth
TBP  TATA-box binding protein
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>$T_m$</td>
<td>melting temperature</td>
</tr>
<tr>
<td>$\mu$</td>
<td>micro</td>
</tr>
<tr>
<td>UFGT</td>
<td>UDP-Glc:flavanoid 3-O-glucosyltransferase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>w/v</td>
<td>weight by volume</td>
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<td>volume by volume</td>
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<tr>
<td>V</td>
<td>Volume</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside</td>
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<td>5-Bromo-4-chloro-3-indolyl $\beta$-D-glucuronide cyclohexylamine salt</td>
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