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

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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 rosea^{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 rosea^{dorsea} petal tissue.

Vascular expression of the *Rosea1* mRNA is indicative of regulation of this gene through sugar or hormonal cues. However, *rosea1*^{dorsea} transcript levels (in rosea^{dorsea}) were much lower than *Rosea1* (wild type). Lowered expression of *rosea1*^{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 *rosea1*^{dorsea}. These changes may lead to instability or the lower expression of the *rosea1*^{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 Rosea1 was analysed by RNAi and bioinformatics-based methods. The presence of potential MADS box binding sites in the intron 2 region of the *Rosea1* allele indicated that *Rosea1* 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 *Rosea1* 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
F3'5'H	Flavanoid 3'5'-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 ß-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
N_2	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

TBE	Tris-Borate-EDTA
T_m	melting temperature
µ	micro
UFGT	UDP-Glc:flavanoid 3-*O*-glucosyltransferase
UV	Ultra Violet
w/v	weight by volume
v/v	volume by volume
V	Volume
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
X-GlcA	5-Bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylamine salt