How the Pigment Stripes Form in Snapdragon (*Antirrhinum majus*) Flowers: a study of the molecular mechanism of venation pigmentation patterning in flowers

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Abstract

Floral stripes are a common pigmentation pattern in plants. Defining the molecular mechanisms of the striped pattern formation will aid understanding of how a gene can be differentially regulated across a population of similar cells. In the venation phenotype of *Antirrhinum majus*, the anthocyanin pigment is typically confined to the adaxial epidermal cells overlaying the petal veins.

To explore how this pattern forms this study focused on the expression and regulation of *Venosa*, a *Myb* regulator of anthocyanin biosynthesis. Pigment complementation experiments demonstrated that the lack of a MYB factor caused the lack of pigment in the cells outside the venation pigmentation domain. An allele of *Venosa* was isolated and identified. It was a mutant version of functional *Venosa* due to the central part being replaced by a transposon. Phenotype / genotype analysis indicated that the venation pigmentation patterning was due to the functional *Venosa*. *In situ* mRNA hybridisation showed that *Venosa* was expressed from the xylem to the adaxial epidermis, and was controlled spatially and quantitatively by a signal associated with the petal veins. *Venosa* expression provided the longitudinal axis for venation pigmentation stripes, and determined the location and intensity of the pigmented cells. Because another factor required for pigmentation, a bHLH factor, is specifically expressed in epidermal cells and it provides the transverse axis. The pigmented stripes are the cross expression domain of these two kinds of factors.

The transcriptional controlling property of a 2.4 kb (relative to the ATG) promoter region of the *Venosa* gene was analysed. The -900 bp fragment was characterised in detail using 5′-end deletion mutagenesis. A heterologous host, tobacco, was used for analysis in stable transgenics. The homologous host, *Antirrhinum*, was used for transient assays. The efficacy and efficiency of different reporter genes (intron-containing GUS, GFP, *Venosa* cDNA and genomic *Venosa*) and enhancement systems (transcriptional enhancer, translational enhancer, inhibitor of post transcriptional gene silencing and a two-step signaling amplification system) for the detection of low-level reporter gene expression were also tested. The strength of expression correlated to the length of the promoter fragment, and expression was detected using deletions down to

-500 bp, although only weak expression was found. This expression was flower specific but not vein related in both plant hosts. No expression was detected in petals of either host with fragments shorter than -500 bp. The results suggest that the fragment from -380 bp to -900 bp positively affected *Venosa* expression at the transcriptional level, but might not be sufficient to define venation. A possibility is that the venation controlling property is negatively controlled at the epigenetic level, such as DNA methylation status and / or chromatin structure.

The role of gibberellin and sugar in the pigment and venation patterning formation of *Antirrhinum* was studied. The results suggest that gibberellin is not required for pigmentation or venation patterning. Convincing evidence on the role of sugar signaling could not be obtained from the experiments, due to the difficulty in separating the impact on pigmentation from other functions of sugars in petal development.

In addition, the *in situ* analysis detected the expression of a gene probably related to aurone biosynthesis that may be a regulatory gene of this biosynthetic pathway.

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Abbreviations

A_{260}	absorbance at 260 nm
A_{600}	absorbance at 600 nm
A	adenine
ANS	anthocyanidin synthase
AS	acetosyringone
ATP	adenosine triphosphate
6-BAP	6-benzylamino purine
bp	base-pairs
^{o}C	degrees Celsius
C	cytosine
CaMV 35S	cauliflower mosaic virus 35S promoter
cDNA	complementary DNA
CHS	chalcone synthase
cm	centimetre
cv	cultivar
ATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DFR	dihydroflavonol 4-reductase
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetracetic acid
EtBr	ethidium bromide
F3H	flavanone 3-hydroxylase
g	gram
G	guanine
GA	gibberellin
GA_3	gibberellic acid
GBV	genomic big venosa

GFP	green fluorescent protein
GMO	genetically modified organism
GSV	genomic small venosa
GUS	ß-glucuronidase
gVenosa	genomic *Venosa*
h	hour
IGUS	intron GUS
IPTG	isopropyl-ß-D-thiogalactoside
Kan	kanamycin
kb	kilo base-pairs
KV	kilo volts
L	litre
LB	Luria-Bertani (media or broth)
M	molar, moles per litre
min	minute
µg	micro gram
mg	milligram
mL	millilitre
µM	micro molar, micro moles per litre
MOPS	3-[*N*-morpholino] propanesulphonic acid
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog Basal Medium
NaHAc	sodium acetate
ng	nanogram
NOS	nopaline synthase
nptII	neomycin phosphotransferase gene
OCS	octopine synthase
PCR	polymerase chain reaction
pmol	pico-molar, pico moles per litre
rATP	riboxyadenosine triphosphate
rCTP	riboxycytidine triphosphate
rGTP	riboxyguanosine triphosphate
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
rUTP	riboxyuradine triphosphate
SDS	sodium dodecyl suphate
SSC	saline sodium citrate buffer
T	thymine
TBE	tris borate EDTA buffer
TBS	tris-buffered saline solution
TE	tris-EDTA buffer
TFs	transcription factors
Tris	tris(hydroxymethyl)aminomethane
Tween20	polyoxyethylenesorbitan monolaurate
U	uracil
V	volts
VEN	*Venosa* promoter deletions
Vv	*Venosa/venosa* heterozygous
vv	*venosa/venosa* homozygous
v/v	volume per volume
w/v	weight per volume
X-Gluc	5'-bromo-4-chloro-3-indoyl-ß-D-glucuronide