FERNANDA MENDES DE REZENDE

**Chemical analysis of color change and transcriptional profile in
flavonoids biosynthesis during flower development of *Tibouchina
pulchra* (Cham.) Cogn.**

**Análises químicas da mudança de cor e perfil transcricional da
biossíntese de flavonoides durante o desenvolvimento floral de
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que de alguma forma contribuíram
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*Gosto das cores, das flores, das estrelas, do verde
das árvores, gosto de observar. A beleza da vida se
esconde por ali, e por mais uma infinidade de
lugares, basta saber, e principalmente, basta querer
enxergar.*

Clarice Lispector

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Resumo

A mudança de cor em flores é um fenômeno generalizado entre as angiospermas, mas pouco compreendido do ponto de vista genético e químico. Este estudo teve como objetivo investigar esse fenômeno em *Tibouchina pulchra*, uma espécie nativa brasileira, cujas flores mudam do branco para rosa escuro. Para alcançar o objetivo, os perfis dos compostos e de expressão de genes da via de biossíntese de flavonoides foram estudados. Utilizando técnicas hifenadas (UPLC-HRMS and NMR), trinta compostos foram quantificados e identificados, sendo: dezesseis descritos pela primeira vez em *T. pulchra*. Além disso, um composto inédito foi identificado. Cinco genes que codificam para enzimas chaves da via foram parcialmente clonados, sequenciados, e os níveis de mRNA foram analisados (RT-qPCR) durante o desenvolvimento das flores. Coletivamente, os dados obtidos demonstram que a mudança de cor em flores de *T. pulchra* é regulada transcionalmente. Além disso, quantificações de metais sugerem que o íon Fe^{3+} esteja relacionado a saturação da cor no estágio rosa escuro.

Palavras-chave: antocianina, ácidos fenólicos, CHS, FLS, ANS

Rezende, F.M. Chemical analysis of color change and transcriptional profile in flavonoids biosynthesis during flower development of *Tibouchina pulchra* (Cham.) Cogn. [thesis]. São Paulo: Instituto de Biociências, Universidade de São Paulo; 2018.

Abstract

Floral color change is a widespread phenomenon in angiosperms, but poorly understood from the genetic and chemical point of view. This thesis aimed to investigate this phenomenon in *Tibouchina pulchra*, a native species whose flowers change from white to dark pink. To reach this goal, flavonoid biosynthetic gene expression and compounds were profiled. By using hyphenated techniques (UPLC-HRMS and NMR), thirty phenolic compounds were quantified and identified, being sixteen described for the first time in *T. pulchra*. Moreover, an inedite compound was also identified. Five key genes of the flavonoid biosynthetic pathway were partially cloned, sequenced, and the mRNA levels were analyzed (RT-qPCR) along flower development. Collectively, the obtained results demonstrated that the flower color change in *T. pulchra* is regulated at transcriptional level. Additionally, a metal quantification suggests that Fe^{3+} ion might influence the saturation of the color at dark pink stage.

Key words: anthocyanin, phenolic acids, CHS, FLS, ANS

List of Abbreviations

[M+H]: molecular ion	DNase: desoxyribonuclease
2-ODD: 2-oxoglutarate/Fe(II)-dependent dioxygenase	Dntp: diphosphate desoribonucleotides
4CL: 4-coumarate:CoA-ligase	DO: diphenol oxidase (DO)
aa: amino acids	DOD: DOPA dioxygenase
AACT: anthocyanidin acyl transferase	DOPA: dihydrophenylalanine
ACT: acyl transferase	DW: dried weight
amu: atomic mass unit	DXP: 1-deoxy-D-xylulose-5-phosphate
ANS: anthocyanidin synthase	DXR: DXP reductoisomerase
AT: <i>Arabidopsis thaliana</i>	DXS: deoxy-D-xylulose synthase
B5GT: betanidin-5-O-glucosyltransferase	DXS: DXP synthase
bbCID: broadband collision induced dissociation	E4P: erythrose 4-phosphate
bHLH: basic helix-loop-helix	EDTA: ethylenediaminetetraacetic acid
BLAST: basic local alignment search tool	ESI: electrospray ionization
Bp: base pair	Eucgr: <i>Eucalyptus grandis</i>
Brara: <i>Brassica rapa</i>	F3'5': flavonoid 3'5'-hydroxylase
C3H: coumarate 3-hydroxylase	F3'H: flavonoid 3'-hydroxylase
C4H: cinnamate 4-Hydroxylase	F3H: flavonoid 3-hydroxylase
CCD: carotenoid cleavage dioxygenase	FDP: farnesyl diphosphate synthase
CD: carotene desaturase	FGT flavonoid glucosyltransferase
CD ₃ OD: deuterated methanol	FLS: flavonol synthase
cD5G2'GlcUT: cyclo-DOPA-5-glucose-2'-O-glucuronyl- transferase	FPP: farnesyl diphosphate
cD5GT: cyclo-DOPA-5-O-glucosyltransferase	FS: flavone synthase
CDNA: complementary DNA	GA3P: glyceraldehyde 3-P
cDOPA: cyclo: DOPA	GDS: GPP synthase reaction
CHI: chalcone isomerase	GGDS: geranylgeranyl diphosphate synthase
CHS: chalcone synthase	GGPP: geranylgeranyl diphosphate
CI: carotene isomerase	GPP: geranyl diphosphate
Co-A: Coenzyme A	HCl: hydrochloric acid
Ct: threshold cycle	HMBC: heteronuclear multiple bond correlation
CTAB: cetyl trimethylammonium bromide	HPLC: high performance liquid chromatography
d: doublet	HRMS: high-resolution mass spectrometry
DAD: diode array detector	HSQC: heteronuclear single quantum coherence
dd: double doublet	IFS: isoflavone synthase
DEPC: diethylpyrocarbonate	IPP: isopentenyl diphosphate
DFR: dihydroflavanol 4-reductase	IPPI: isopentenyl diphosphate isomerase
DMAPP: dimethylallyl diphosphate	IPTG: isopropyl β-D-1-thiogalactopyranoside
DMSO-d ₆ : deuterated dimethyl sulfoxide	IspH: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase
DNA: desoxyribonucleic acid	J: coupling constant

LB: Luria Bertani growth medium	RNA: ribonucleic acid
LCYB: Lycopene B-Cyclase	RNAi: RNA interference
LCYE: Lycopene B-Cyclase	RT: retention time
m: multiplet	RT-Qpcr: reverse transcriptase quantitative polymerase chain reaction
MAL: malonate pathway	s: singlet
Me: methyl group	S1: stage one of <i>T. pulchra</i> flower
Medtr: <i>Mendicago trunculata</i>	S2: stage two of <i>T. pulchra</i> flower
MeOH: methanol	S3: stage three of <i>T. pulchra</i> flower
MEP: 2-C-methyl-D-erythritol 4- phosphate	S4: stage four of <i>T. pulchra</i> flower
MEP: methylerythritol phosphate pathway	SKM: shikimate pathway
MEV: mevalonate pathway	sl: large singlet
miRNA: micro RNA	SOC: super optimal broth
mRNA: messenger RNA	Soly: <i>Solanum lycopersicum</i>
MS: mass spectrometry	tasiRNA: trans-acting short-interfering RNA
MYB: myeloblastosis	TOH: tyrosine hydroxylase
n: nucleotide	Tp: <i>Trifolium pratense</i>
NMR: nuclear magnetic resonance	UPLC: ultra performance liquid chromatography
NS: neoxanthin synthase	UV/Vis: ultraviolet/visible
OMT: O-methyl transferase	WDRs: tryptophan-aspartic acid dipeptide repeat
P450: Cytochrome P450 Monooxygenase	X-Gal: 5-bromo-4-chloro-3-indoxyl- β -D-galactopyranoside
PAD: photodiode array detector	ZE: zeaxanthin epoxidase
PAL: phenylalanine ammonia lyase	β -CO: β -carotene oxygenase
PCR: polymerase chain reaction	B-RH: B-Ring Hydroxylase
PD: phytoene desaturase	δ : chemical shift
PEP: phosphoenolpyruvate	E-RH: E-ring hydroxylase
ppm: parts per million	
PS: phytoene synthase	
QTOF: quadrupole time-of-flight	

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Chapter 1- Plant pigments involved in floral color.

1. INTRODUCTION

Flower colors are important in the interaction between plants and their pollinators. Since Darwin evolutionary theory, biologists have studied the role of floral variation in plant reproductive fitness and its influence in pollination. Flower colors define pollination syndromes, which are associated with pollinator groups such as bees, moths, butterflies, bats, birds, beetles, flies, and others (Fenster *et al.* 2004; Yan *et al.* 2016). The coevolution of pollinators and angiosperm drives the floral diversity (*e.g.* flower morphology, color, scent, nectar quantity and nectar quality) (Van der Niet *et al.* 2014), and this resulted in reproductive isolation and plant speciation (Kay & Sargent 2009).

Interestingly, pollinators have different color vision spectra and, due to the diverse chemical nature of molecules, the differential accumulation of specific metabolites leads to changes in pollinator preferences, which may not appear evident to the human eye (Grotewold 2006). For example, bee-pollinated flowers are often blue or yellow but rarely red, which may appear black to bees (Grotewold 2016; Papiorek *et al.* 2016).

Furthermore, flower coloring is an important aesthetic factor for the ornamental plant market since it directly influences the commercial value of flowers (Zhao & Tao 2015). Gregor Mendel, who established the fundamental laws of genetics, was interested in plant breeding and succeeded in obtaining a new variety of *Fuchsia* (Onagraceae, Myrtales) with a novel color, which rendered him a prize for his agronomic research (Freire-Maia 1995). The advent of genetic engineering tools brings new insights to basic research and breeding, aiming to obtain different coloring patterns (*i.e.* more lasting or vibrant), or completely different colors from those of the wild type genotype. This is the case of the first genetically modified commercial flower species, *Dianthus* (Caryophyllaceae, Caryophyllales), which display a wide variation of colors ranging from white to red (Tanaka *et al.* 2005).

According to the European Commission, in 2012, the European Union assumed 42.6% of the total world flower and ornamental plant production, followed by China, USA and Japan with 15.5%, 11.1% and 9.5%, respectively. Brazil is the ninth in the ranking with 1.71% of the world market (Neves & Pinto 2015). In Brazil, there is increasing investment and attention to flower and ornamental plant production market. Since 1970, the floriculture has a commercial

and professional character, promoted mainly by Japanese and Dutch immigrants. The creation of the Brazilian Institute of Floriculture (IBRAFLO) in 1994, was an important enterprise to integrate and politically represent the Brazilian's interests at national and international levels. In 2010, a strategic agenda was created focused on encouraging and supporting the development and growth of the flower and ornamental plant domestic market (AEFP 2011). Since 2012, the increasing of this sector, with 6.17% per year, exposes the success of these strategies (Neves & Pinto 2015). Brazilian flora provides an invaluable diversity of native ornamental species to be exploited highlighting the economic potential of this market for the country's development.

The color of the flowers is one of the best-studied floral traits in terms of its genetic and chemical bases, as well as its ecological significance. Regardless the chlorophyll, there are three types of pigments present in vegetal tissues and all confers color to flowers: carotenoids, betalains and flavonoids (specially anthocyanins). These secondary metabolites are originated from products of the primary metabolism by the plastidial shikimate (SKM) and methylerythritol phosphate (MEP) pathways, and the cytosolic malonate (MAL) pathway. Carotenoids are produced by MEP, SKM originates betalains, while intermediates of SKM and MAL condensate to produce flavonoids. In this chapter, we review the biosynthesis of these compounds and the current knowledge about spatial and temporal flower color change, an unusual, but widespread phenomenon in angiosperms.

1.1. Carotenoids

Carotenoids, also known as tetraterpenoids (C_{40}), belong to terpenoid class of secondary metabolites. They are lipid soluble substances produced by all photosynthetic organisms (plants, algae and bacteria), likewise some non-photosynthetic bacteria and fungi (Hirschberg *et al.* 1997; Li *et al.* 2001). In green tissues, carotenoids act as components of the light harvesting machinery and photo-protectants from oxidative damage, being essential players for photosynthetic activity (Bartley & Scolnik 1995; Ronen *et al.* 1999; Davies 2004). Yet, in many flowers and fruits, after chloroplasts to chromoplast transition, carotenoids act as pollinator and seed dispersal attractants (Ronen *et al.* 1999; Niyogi *et al.* 2001; Lao *et al.* 2011).

Since 19th century, carotenoids, together with other natural pigments, attracted the attention of organic chemists. β -carotene was the first carotenoid isolated in 1817 and, in 1937, xanthophylls were identified as the pigments involved in autumnal leaf color (Isler 1971).

Furthermore, carotenoids are essential components of human diets, providing precursors for the biosynthesis of vitamin A (Krinsky & Johnson 2005). They also serve as antioxidants, reducing age related degeneration of the eye and lowering the risk of cancer and cardiovascular diseases (Sandmann *et al.* 2006; Johnson & Krinsky 2009; Ford & Erdman 2012).

In general, yellow and orange tones shown by tissue/organ plants are conferred by carotenoids (Tanaka 2010), especially in flowers. Carotenoids can also coexist with anthocyanins resulting in brown and bronze tones despite of their contribution for red colors (Forkmann 1991; Nisar *et al.* 2015).

The building blocks of all terpenoids are isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Chappell *et al.* 1995), being synthesized by two distinct pathways: mevalonate (MEV) and MEP, cytosolic and plastidial localized, respectively (Lichtenthaler 1999; Eisenreich *et al.* 2001; Rodriguez-Concepcion & Boronat 2002). However, the exchange of intermediates between the pathways is well documented (Dudavera *et al.* 2006).

MEV pathway supplies most of the C₅ units for the biosynthesis of C₁₅ (sesquiterpenes), C₃₀ (triterpenes and sterols), and larger compounds (dolichols), substances whose later biosynthetic steps take place in cytosol. However, plastidial localized terpenoids, such as C₅ (isoprene), C₁₀ (monoterpenes), C₂₀ (diterpenes), C₃₀ (triterpenes) and C₄₀ carotenoids are synthesized *via* MEP (Lichtenthaler *et al.* 1997; Milborrow & Lee 1998; Matusova *et al.* 2005; Rodriguez-Concepcion 2010; Buchanan *et al.* 2015). The MEP pathway (Figure 1.1) initiates with glyceraldehyde 3-phosphate (GA3P) and pyruvic acid that by the action of the DEOXY-D-XYLULOSE SYNTHASE (DXS) form deoxy-D-xylulose 5-phosphate (DXP). Subsequently intramolecular rearrangement and reduction of DXP by the DXP REDUCTOISOMERASE (DXR) leads MEP formation. After some steps IPP and DMAPP are formed, and undergo sequential condensation reactions to form C₂₀ geranylgeranyl diphosphate (GGPP), precursor of carotenoid biosynthesis. The condensation of two molecules of GGPP, tail-to-tail, results in phytoene, which is further converted in lycopene by a series of desaturations and isomerizations by the action of PHYTOENE DESATURASE (PD), CAROTENE DESATURASE (CD) and CAROTENE ISOMERASE (CI). Lycopene represents a branch point in carotenoid pathway that is enzyme-mediated cyclized by LYCOPENE CYCLASES (LCY ϵ , LCY β), to produce α -carotene or β -carotene (Nisar *et al.* 2015). These two compounds are precursors of the oxygenated carotenoids, xanthophylls (*e.g.* lutein, violaxanthin and zeaxanthin), which are produced by the

action of β -RING HYDROXYLASE (β -RH) and ϵ -RING HYDROXYLASE (ϵ -RH) (Finkelstein 2013; Nisar *et al.* 2015).

The enzyme encoding genes involved in carotenogenesis have been identified in many plant species, while its regulation has been addressed by several studies (Fraser & Bramley 2004; Tanaka *et al.* 2008; Li & Yuan, 2013; Liu *et al.* 2015a). Some steps are regulated by light and chloroplast redox status (Jahns & Holzwarth 2012; Llorente *et al.* 2016). In Solanaceae crop species (*e.g.* tomato fruit) and *Arabidopsis thaliana* (L.) Heynh model plant, transcription factors of carotenoid biosynthetic genes were well characterized (*e.g.* PHYTOCHROME INTERACTING FACTOR 1a, RIPENING INHIBITOR, etc) (Toledo-Ortiz *et al.* 2010; Martel *et al.* 2011), demonstrating that the accumulation of carotenoids is transcriptionally regulated. Red-colored flowers due to the accumulation of carotenoids are rare among angiosperms and even scarcer are the studies about their biosynthetic regulation (Ohmiya 2011).

Figure 1.1. Simplified schematic representation of plant pigment biosynthetic pathways. Primary metabolism, **carotenoid**, **flavonoid** and **betalain** pathways are highlighted in green, orange, red, and purple, respectively. In blue boxes are the names of the main intermediate for each pathway: SKM (shikimate), MEP (methyl-D-erythritol 4-P), and MAL (malonate). Abbreviations indicate the following metabolites and enzymes: 1-deoxy-D-xylulose-5-P (DXP), 4-HYDROXY-3-METHYLBUT-2-ENYL DIPHOSPHATE REDUCTASE (IspH), ACYL TRANSFERASE (ACT), ANTHOCYANIDIN ACYL TRANSFERASE (AACT), ANTHOCYANIDIN SYNTHASE (ANS), AROGENATE DEHYDRATASE (AD), AROGENATE DEHYDROGENASE (ADH), CAFFEIC ACID O-METHYLTRANSFERASE (COMT), CAROTENE ISOMERASE (CI), CHALCONE SYNTHASE (CHS), CHALCONE ISOMERASE (CHI), CHALCONE REDUCTASE (CHR), CHORISMATE MUTASE (CM), CINNAMATE 4-HYDROXYLASE (C4H), Coenzyme A (CoA), COUMARATE 3-HYDROXYLASE (C3H), cyclo-DOPA (cDOPA), DIHYDROFLAVANOL 4-REDUCTASE (DFR), dihydrophenylalanine (DOPA), dimethylallyl diphosphate (DMAPP), DIPHENOL OXIDASE (DO), DOPA DIOXYGENASE (DOD), DXP REDUCTOISOMERASE (DXR), DXP SYNTHASE (DXS), erythrose 4-phosphate (E4P), farnesyl diphosphate (FPP), FARNESYL DIPHOSPHATE SYNTHASE (FDS), FLAVANONE 3'-HYDROXYLASE (F3'H), FLAVANONE 3'-HYDROXYLASE (F3'H), FLAVANONE 3-HYDROXYLASE (F3H), FLAVONE SYNTHASE (FS), FLAVONOID GLYCOSYL TRANSFERASE (FGT), FLAVONOL SYNTHASE (FLS), geranyl diphosphate (GPP), geranylgeranyl diphosphate (GGPP), GERANYLGERANYL DIPHOSPHATE SYNTHASE (GGDS), glyceraldehyde 3-phosphate (GA3P), GPP SYNTHASE (GDS), Coenzyme A (HSCoA) ISOFLAVONE SYNTHASE (IFS), ISOPENTENYL DIPHOSPHATE ISOMERASE (IPPI), isopentenyl

diphosphate (**IPP**), LYCOPENE β -CYCLASE (**LCY β**), LYCOPENE ϵ -CYCLASE (**LCY ϵ**), NEOXANTHIN SYNTHASE (**NS**), O-METHYLTRANSFERASE (OMT), PHENYLALANINE AMMONIA LYASE (**PAL**), PHENYLPYRUVATE AMINOTRANSFERASE (**PPAT**) phosphoenolpyruvate (**PEP**), PHYTOENE DESATURASE (**PD**), PHYTOENE SYNTHASE (**PS**), PREPHENATE AMINOTRANSFERASE (**PAT**) PREPHENATE DEHYDROGENASE (**PDH**), SHIKIMATE DEHYDROGENASE (**SDH**), TYROSINE AMMONIA LYASE (**TAL**), TYROSINE OXYDASE HYDROXYLASE (**TOH**), UDP-GLUCOSYLTRANSFERASE (**UGT**), ZEAXANTHIN EPOXIDASE (**ZE**), B-CAROTENE OXYGENASE (**β -CO**), β -RING HYDROXYLASE (**β -RH**), ϵ -RING HYDROXYLASE (**ϵ -RH**). Adapted from Andersen & Markham (2005), Dewick (2009), Khan & Giridhar (2015), and Nisar *et al* (2015).

1.2. Betalains

Betalains are pigments possessing a nitrogenous core structure. They confer colors from red to violet (betacyanins) or yellow to orange (betaxanthins) and are exclusively present in members of some families of Caryophyllales order. This well-defined clade comprises 38 families being 17 betalain accumulators (Strack *et al.* 2003; Grotewold 2006; APG 2016). Betalains are considered chemosystematics markers of the core Caryophyllales (Slimen *et al.* 2017), where approximately 75 distinct compounds were identified (Khan & Giridhar 2015). Interestingly, within core Caryophyllales, only two families (*i.e.* Caryophyllaceae and Molluginaceae) are known to produce anthocyanin as the noncore Caryophyllales (Brockington *et al.* 2011).

As well as many other naturally occurring compounds, betalains present chemical instability related to several factors, such as light, temperature, oxygen, pH value, water content, and storage conditions (Gonçalves *et al.* 2012; Fernández-Lopez *et al.* 2013; Molina *et al.* 2014; Otálora *et al.* 2016).

Betalains are derived from the amino acid L-tyrosine, an aromatic amino acid synthesized by the SKM plastidial pathway (Figure 1.1). The primary substrates for this pathway are the phosphoenolpyruvate (PEP- a glycolytic intermediate) and the erythrose 4-phosphate (E4P- from the pentose phosphate cycle). Chorismic and prephenic acids are key intermediates that lead to the synthesis of the aromatic amino acids: phenylalanine, tryptophan, and tyrosine. These amino acids are involved in aromatic alkaloids and phenolic compounds pathways. In monocots, tyrosine is the precursor for phenolic compounds synthesis, while in eudicots, the universal precursor of numerous phenolic compounds (*e.g.* phenylpropanoids, lignans, lignins, stilbenes and flavonoids) is the phenylalanine (Maeda & Dudavera 2012).

Betalain synthesis starts with dihydrophenylalanine (DOPA) formation catalyzed by the TYROSINE OXYDASE HYDROXYLASE (TOH). DOPA is an important precursor not only of betalains but also of other secondary metabolites in plants (Tanaka 2008). The cleavage of

DOPA mediated by DOPA 4,5-DIOXYGENASE (DOD) followed by non-enzymatical rearrangements result in the formation of betalamic acid, the core structure of betalains (Khan & Giridhar 2015). Betalamic acid is the chromophore molecule of both betacyanins (violet) and betaxanthins (yellow) (Tanaka 2008). Its condensation with cyclo-DOPA (cDOPA) and glucosyl derivatives, amino acids, and amine groups leads to the formation of these two categories of betalains: betacyanins and betaxanthins (Khan & Giridhar 2015) (Figure 1.1).

Betaxanthins were structurally identified in *Beta vulgaris* L. (Amaranthaceae), *Portulaca grandiflora* Hook. (Portulacaceae), *Mirabilis jalapa* L. (Nyctaginaceae), *Glottiphyllum longum* (Haw.) N.E.Br. (Aizoaceae), *Rivina humilis* L. (Phytolacaceae), *Amaranthus tricolor* L. (Amaranthaceae), among other species. Moreover, betacyanins were found in *Celosia argentea* L. (Amaranthaceae), *Iresine herbstii* Hook. (Amaranthaceae), *Bougainvillea glabra* Choisy (Nyctaginaceae), and *Gomphrena globose* L. (Amaranthaceae) (Khan & Giridhar 2015).

Betalains and anthocyanins seem to be mutually exclusive pigments because there are no evidences of the occurrence of these two pigments in the same plant. These two classes of compounds play equal biological function, since both confer similar color to flowers and fruits, and accumulate in response to stress signals. The functional and biochemical similarities among betalains and anthocyanins suggest similar biosynthetic regulatory mechanisms (Stafford 1994; Brockington *et al.* 2011). Studies with *Phytolacca americana* Z. (Phytolacaceae) (Takahashi *et al.* 2009) demonstrated that DOD encoding gene is transcriptionally regulated. The identification of MYELOBLASTOSIS (MYB) and BASIC HELIX–LOOP–HELIX (bHLH) transcription factor responsive elements in *P. americana* DOD promoter region suggest that these proteins regulate betalain biosynthesis (Takahashi *et al.* 2009). Interestingly, MYB and bHLH, together with TRYPTOPHAN-ASPARTIC ACID DIPEPTIDE REPEAT proteins (WDRs) are transcription factors that interact among each other to form the MBW complex, which has been extensively demonstrated to regulate flavonoid biosynthesis (Hichri *et al.* 2011). Recently, the first MYB-like regulatory gene for betalain pathway, *BvMYB1*, has been identified in *Beta vulgaris*, however, unlike anthocyanin-related MYBs, the product of this gene does not interact with bHLH members of the MBW complex. Phylogenetic analyses indicated that *BvMYB1* and anthocyanin MYBs of *Antirrhinum majus* L. (Plantaginaceae, Lamiales) (*AmROSEA*) and *A. thaliana* (*AtPAP1*) (Solanaceae, Solanales) probably derive from a common ancestor gene (Hatléstad *et al.* 2014).

Recent studies on comparative genetics of betalain producing taxa revealed another possible explanation for the mutual exclusion between anthocyanins and betalains. The promoter region of *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) and *ANTHOCYANIDIN SYNTHASE* (*ANS*) are different in anthocyanin and betalain producing species. Both genes are expressed in betalain producing taxa, but they do not lead to anthocyanin production (Shimada *et al.* 2005, 2007). It has been proposed that, even when the putative MYB and bHLH transcriptional factors for anthocyanin biosynthesis are present and bind to the *DFR* and *ANS* promoters of the Caryophyllales, other factors involved in the activation of these genes might be missing in anthocyanin non-producing plants (Broun 2005; Ramsay *et al.* 2005; Shimada *et al.* 2007). The other possible explanation is the presence of a repressor-binding site in Caryophyllales promoters (Shimada *et al.* 2007). Despite of advances in this understanding, the evolution of this pathway and the mechanism behind the mutual exclusivity of betalains and anthocyanins has not been resolved (Sakuta 2014).

1.3. Anthocyanins

Among angiosperms, flavonoids, specially anthocyanins, are the predominant floral pigments (Winkel-Shirley 2001; Tanaka *et al.* 2010). These pigments are responsible for the red/orange to violet/blue coloration of fruits and flowers according to the vacuole pH (Katsumoto *et al.* 2007). Similar to carotenoids, anthocyanins act as photoprotectants, due to their UV absorption spectra and high antioxidant activity. In general, in non-reproductive tissues, anthocyanins are accumulated in response to biotic and abiotic stresses, such as pathogens, high/low temperatures, high light, UV-B radiation, drought, nutrient deficiencies, high ozone concentration, etc. (Chalker-Scott 1999; Feild *et al.* 2001; Neill *et al.* 2002; Oberbauer & Starr 2002; Steyn *et al.* 2002; Takos *et al.* 2006; Gould *et al.* 2008; Rezende & Furlan 2009).

Anthocyanins are synthesized by the combination of precursors from the SKM and MAL pathways, being the terminal branch of the highly conserved flavonoid pathway (Holton & Cornish 1995). Flavonoids constitute a relatively diverse family of aromatic molecules derived from L-phenylalanine (Winkel-Shirley 2001). Flavonoid synthesis is initiated by the action of the CHALCONE SYNTHASE (*CHS*) that catalyzes the condensation of *p*-coumaroyl-CoA (SKM) with three molecules of malonyl-CoA (MAL- fatty acid pathway), resulting in the formation of a tetrahydroxylalcohol. This compound is cyclized producing chalcone, precursor of all classes of

flavonoids including flavonols, flavones, flavandiols, dihydroflavonols, proanthocyanidins, isoflavonoids and anthocyanins (Dewick 2009).

Flavones are formed from the C-ring dehydrogenation of flavanones, a class of flavonoids formed by chalcone isomerization mediated by the enzyme CHALCONE ISOMERASE (CHI), which forms a third ring, characteristic in the C₆C₃C₆ flavonoid basic structure (Dewick 2009).

Dihydroflavonol is the branching point for the synthesis of flavonols and anthocyanins by FLAVONOL SYNTHASE (FLS) and DFR, respectively (Cooper-Driver 2001; Saito & Yamazaki 2002; Dewick 2009; Buchanan *et al.* 2015). Anthocyanin synthesis further requires the activity of ANS, FLAVONOID GLYCOSYL TRANSFERASE (FGT), ANTHOCYANIDIN ACYL TRANSFERASE (ACT) and O-METHYL TRANSFERASE (OMT). Anthocyanidins are the aglycones and chromophores of anthocyanins (glycosylated compounds), probably the best-known and most studied group of flavonoids.

In nature, anthocyanins provide a wide range of colors. Out of the twenty-three described anthocyanidins, six are particularly abundant: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin. The difference in color tones is determined by the substitution patterns in the core structure of the B-ring (*e.g.* hydroxyl and methoxyl) and in the A and C-ring (*e.g.* glycosylations and acylations) (Castañeda-Ovando *et al.* 2009, Trouwillas *et al.* 2016).

When considering their structures, studies on anthocyanins have demonstrated that they can exist in various colored and colorless forms; the predominant form is largely determined by pH. At acidic pH (< pH 2.5), the dominant form is the colored flavylium cation form, however, about pH 3 most anthocyanins undergo nucleophilic attack by water with subsequent loss of a proton resulting in the colorless or pale-yellow hemiacetal (or hemiketal) form. The hemiacetal form can then undergo ring-opening tautomerization to the *cis*-hydroxychalcone or (*Z*)-hydroxychalcone, which can isomerize thermally or photochemically to the *trans*-chalcone or (*E*)-chalcone, both of which are also colorless or pale-yellow (Silva *et al.* 2016). Additionally, anthocyanins with two or more *ortho*-hydroxyl groups in the B-ring form complexes with divalent and trivalent metal cations, such as Al³⁺, Fe²⁺/Fe³⁺, and Mg²⁺, which results in color changes (Buchweitz *et al.* 2001; Yoshida *et al.* 2009; Soumille *et al.*, 2013; Sigurdson *et al.* 2014).

Two categories of genes are involved in the biosynthesis of anthocyanins (Martin *et al.* 1991; Jackson *et al.* 1992): structural enzyme encoding genes, such as *PHENYLALANINE AMONIA LIASE (PAL)*, *CINNAMATE 4-HYDROXYLASE (C4H)*, *CHS*, *FLAVANONE 3-HYDROXYLASE*

(*F3H*), *DFR*, *ANS*, and *FGT*; and regulatory transcription factor encoding genes, basically MYB, bHLH, and WDR proteins, which coregulate the expression of the structural genes (Uematsu *et al.* 2014). Maize (*Zea mays* L., Poaceae, Poales), snapdragon (*A. majus*) and petunia (*Petunia hybrid* Vilm., Solanaceae, Solanales) are the major model species for the study of flavonoid biosynthesis, from which several structural and regulatory genes have been characterized (Holton & Cornish 1995; Mol *et al.* 1998; Whinkel-Shirley 2001; Dhar *et al.* 2014). Moreover, anthocyanin biosynthesis and regulation have also been studied in other plants, such as *Solanum tuberosum* L. (potato) and *S. melongena* L. (eggplant), Solanaceae (Solanales), and *A. thaliana*, etc. (Jeon *et al.* 1996; Holton & Cornish 1997; Quattrocchio *et al.* 1999; Noda *et al.* 2000; Jung *et al.* 2005; Katsumoto *et al.* 2007; Chaudhary & Mukhopadhyay 2012).

Over the past 150 years, flavonoids have featured in major scientific breakthroughs, from Mendel's elucidation of genetic bases, where seed coat color was one of the major characters followed in his experiments with peas (*Pisum sativum* L., Fabaceae, Fabales), to McClintock's discovery of transposable elements, which move in and out of flavonoid biosynthetic genes expressed in colored maize kernels (Whinkel-Shirley 2001). Flavonoid pathway also helped to clarify the phenomenon of transgene co-suppression. Aiming to intensify the violet pigmentation, an extra copy of *CHS* was introduced in *P. hybrida* resulting in totally white flowers. Expression profile analysis showed that in these transgenic plants neither the transgene nor the endogenous copy of the gene were expressed (Napoli *et al.* 1990).

Few studies regarding RNAi-based regulation of flavonoid biosynthetic pathway can be found, but certainly it will be a research focus of interest in the future. In *A. thaliana*, it was reported the regulation of MYB anthocyanin regulators by miRNAs and tasiRNA (Rajagopalan *et al.* 2006; Hsieh *et al.* 2009; Gou *et al.* 2011).

Recently, additional levels of regulation have been reported to modulate the production of anthocyanins. These include the post-translational modifications of anthocyanin transcription factors (Xie *et al.* 2012; Ye *et al.* 2012; Maier *et al.* 2013; Patra *et al.* 2013), chromatin remodeling (Hernandez *et al.* 2007), and the identification of repressor proteins that limit expression of the anthocyanin biosynthetic genes (Aharoni *et al.* 2001; Dubos *et al.* 2008; Matsui *et al.* 2008; Yuan *et al.* 2013).

Despite vacuolar pH, metal complexation and biosynthetic regulations, copigmentation influence the color of plant organs (Tanaka 2010). Copigmentation can be defined as the formation of noncovalent complexes involving an anthocyanin or anthocyanin derived pigment

and a copigment (in the presence or absence of metal ions), and the subsequent changes in optical properties of the pigment. There are over ten-thousand compounds that could potentially serve as copigments but, in any case, they should have (i) sufficiently extended π -conjugated systems, which are supposed to favor π - π stacking interactions and (ii) hydrogen bond donor/acceptor groups such as OH and C=O groups (Trouwillas *et al.* 2016). The major natural copigments are hydrolyzable tannins, flavonoids, and phenolic acids. Some flavonoid classes are copigments (flavonols, flavones, flavanols, and even dihydroflavonols), which appear to be the most efficient due to extension of their π -conjugation over their entire tricyclic core structure (rings A, B, and C), being the flavones and flavonols (*e.g.* quercetin, kaempferol, isoquercitrin, and rutin) the most efficient among them. Hydroxycinnamic acids and derivatives such as caffeic, *p*-coumaric, ferulic, sinapic, chlorogenic, and cactaric acids are commonly described as relatively efficient copigments as well (Trouwillas *et al.* 2016). Some non-phenolic copigments have also been described, including alkaloids, amino acids, organic acids, nucleotides, and polysaccharides (He *et al.* 2012), but their efficiency is usually lower than polyphenols. It remains unclear whether these compounds can significantly contribute to color expression provided by anthocyanins (Trouwillas *et al.* 2016).

2. OBJECTIVES

The overall aim of this thesis was to explore the phenomenon of flower color change in *Tibouchina pulchra* by the chemical characterization of flower extracts and the study of the regulatory mechanisms involved in this temporal phenotypic change. Results were organized in the following to chapters:

Chapter 2: Chemical characterization of *Tibouchina Pulchra* (Cham.) Cogn. flowers.

Chapter two describes the chemical characterization of petal pigments by isolation procedures and spectroscopic analysis (UV-Vis absorption spectra, mass spectra and nuclear magnetic resonance).

Chapter 3: The Regulation of color change in *Tibouchina pulchra* flowers.

In this chapter, the goal was to investigate whether the color change is determined either by the differential accumulation of flavonoid derivatives and/or metals along the different stages of floral development. In order to investigate the putative transcriptional regulation of the biosynthetic enzyme encoding genes, key genes of the flavonoid biosynthesis were cloned and the transcript accumulation patterns profiled along floral development.

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Chapter 2 – Chemical characterization of *Tibouchina pulchra* (Cham.) Cogn. flowers.

1. INTRODUCTION

Tibouchina Aubl. is one of the most representative genera within Melastomataceae A. Juss., the fifth largest angiosperm family in Brazil (BFG 2015). This family contains approximately 170 genera and 4,500 species with a pantropical distribution, but particularly rich and dominant in the Neotropics with about 3,000 species, being 1,367 Brazilian natives. Melastomataceae species can be easily recognized among eudicots by their leaves with characteristic acrodromous venation. It contains thirteen well differentiated tribes (APG 2016).

Recently, a study that addressed the reproductive strategies within three tribes (*i.e.* Miconieae, Microlicieae and Melastomeae) found that Miconieae encompasses pollinator independent and fleshy fruit bearing species, mainly bird dispersed. On the other hand, in Microlicieae and Melastomeae, although the dispersion is abiotic (dry fruit), the breeding strategy depends on pollinators, mainly bees (Brito *et al.* 2017). By means nuclear and plastid markers, a molecular phylogenetic analysis demonstrated that *Tibouchina*, the largest genus in Melastomeae, is polyphyletic once some species grouped together with other genera, such as *Brachyotum*, *Bucquetia*, *Castratella*, *Centradenia*, *Chaetolepis*, *Heterocentron*, *Itatiaia*, *Microlepis*, *Monochaetum*, *Pilocosta*, *Svitramia*, and *Tibouchinopsis*, which are all monophyletic (Michelangeli *et al.* 2013). Moreover, *Tibouchina* has several plesiomorphic characters as the core Melastomeae: tetramerous or pentamerous flowers that show anthers with developed pedoconnectives (*i.e.* the connective tissue below the anther locules) and short bilobed ventral anther appendages. These data clearly expose that *Tibouchina* phylogeny is unsolved and it has been proposed that either the genus is recognized as much-expanded, although no morphological synapomorphies for this clade were identified, or all the other genera could be maintained, while *Tibouchina* should be divided into smaller and diagnosable units (Michelangeli *et al.* 2013).

Tibouchina has approximately 460 species (Clausing & Renner 2001, Tropicos 2017, Guimarães 2014). High level of endemism was verified for Brazilian species, being 145 out of the 166 reported considered restricted to the country, occurring in Atlantic rainforest and Cerrado (BFG 2015, Guimarães 2016). Studies aiming to describe the biological activities of

Tibouchina species have shown promising results. Jiménez *et al.* (2015) reported the use of *Tibouchina kingii* Wurdack in traditional medicine due to its anti-inflammatory properties and demonstrated high antioxidant potential of the aqueous leaf extract. *T. pereirae* Brade & Markgr also exhibited antioxidant activity by free radical scavenging assay and antinociceptive effect of *n*-hexanic extract (Dias *et al.* 2016). Low antimicrobial activity was found in extracts of *Tibouchina candolleana* Cong. (Santos *et al.* 2012). Additionally, isolated compounds (*i.e.* 2,8-dihydroxy-7H-furo[2,3-f] chromen-7-one and isoquercitrin) from *Tibouchina paratropica* (Griseb.) Cong. showed antibacterial and antifungal activity. Interestingly, 2,8-dihydroxy-7H-furo[2,3-f] chromen-7-one also displayed potent antiparasitic activity against *Leishmania donovani* (Tracanna 2015). Antifungal (Niño *et al.* 2003, Kuster *et al.* 2009), anticancer (Jones *et al.* 1981) and antiparasitic activities (Singha *et al.* 1992, Mpalantinos *et al.* 1998, Cunha *et al.* 2009) have also been reported for the genus.

Phytochemical studies in eleven species of *Tibouchina* (*T. candolleana*, *T. ciliares* (Vent.) Cong., *T. grandiflora* Cong., *T. lepidota* (Bonpl.) Baill., *T. multiflora* Cong., *T. paratropica*, *T. pereirae*, *T. pulchra*, *T. semidecantra* (Mart & Schrank ex DC.) Cong., *T. urvilleana* (DC.) Cong., *T. granulosa* (Desr.) Cong.) reported the presence of several natural products, such as flavonoids (flavonol glycosides and anthocyanins), isoflavonoids, phenolic derivatives, tannins and triterpenes (Table 2.1). Structural elucidation by Nuclear Magnetic Resonance (NMR) was performed for some triterpenoids, tannins, flavonols, and anthocyanins in *T. urvilleana* and *T. lepidota* (Terahara *et al.* 1993, Pérez-Castorena 2014, Hendra & Keller 2016). As reviewed above, few studies have addressed the chemical profile of *Tibouchina* species, which remains largely elusive.

Table 2.1- Natural products reported for *Tibouchina* species.

Species	Class of metabolite	Compound	Organ	Reference
<i>T. candolleana</i>	triterpene/ flavone/ isoflavonoid	α - and β -amyrin, β - sitosterol, ursolic and oleanolic acids/ luteolin/ genistein	aerial parts	Dos Santos <i>et al.</i> 2012
<i>T. ciliaris</i>	flavonol	kaempferol 7- <i>O-p</i> -coumaroyl, quercetin 3- <i>O</i> -rhamnopyranoside, quercetin 3- <i>O</i> -arabnoside	leaves	Colorado <i>et al.</i> 2007
<i>T. grandiflora</i>	flavonol/ anthocyanin	quercetin 3- <i>O</i> - β -D-glucuronide, quercetin 3- <i>O</i> - β -D-glucopyranoside, quercetin 3- <i>O</i> - β -D-galactopyranoside, quercetin 3- <i>O</i> - α -L-rhamnopyranoside, quercetin 3- <i>O</i> - β -L-arabinopyranoside, quercetin 3- <i>O</i> - β -D-(6"- <i>p</i> -coumaroyl)-glucopyranoside/ peonidin 3-sophoroside, peonidin 3-sambubioside, malvidin 3,5-diglucose, malvidin 3-(<i>p</i> -coumaroyl)-sambubioside-5-glucoside	leaves/ flowers	Kuster <i>et al.</i> 2009, Bobbio <i>et al.</i> 1985
<i>T. granulosa</i>	flavonol/ flavone/ proanthocyanidin/ anthocyanin	Isorhamnetin 3- <i>O</i> -glucuronide, isorhamnetin 3- <i>O</i> -di-glucoside, isorhamnetin 3- <i>O</i> -rutinoside, quercetin 3-(<i>O</i> -galloyl)-hexoside/ hispidulin 7- <i>O</i> -glucoside/ B-type procyanidin monomer, dimer, trimer and pentamer/ petunidin, pelargonidin	flowers/ leaves	Okumura <i>et al.</i> 2002, Sobrinho <i>et al.</i> 2017
<i>T. lepidota</i>	tannin/ flavonol/ anthocyanin	gallic acid, 2,3,5-trihydroxybenzoic acid/ quercetin 3- <i>O</i> -arabnoside, quercetin, quercetin 3-glucoside, isorhamnetin 3-rutinoside/ malvidin 3-(<i>p</i> -coumaryl-glucoside)-5-(acetyl-xyloside)	flowers	Hendra & Keller 2016
<i>T. multiflora</i>	tannin	nobotanins O and P	leaves	Yoshida <i>et al.</i> 1999,
<i>T. paratropica</i>	phenolic derivative/ flavonol	2,8-dihydroxy-7H-furo (2,3-f)-chromen-7-one/ isoquercitrin	aerial parts	Tracanna <i>et al.</i> 2015
<i>T. pereirae</i>	flavonol	Isorhamnetin 3- <i>O</i> -galloyl-glucoside, isorhamnetin-3- <i>O</i> -glucoside, kaempferol 3- <i>O</i> -rutinoside, quercetin 3- <i>O</i> -(galloyl)-glucoside, rutin	aerial parts	Dias 2013
<i>T. pulchra</i>	flavonol/ tannin	kaempferol 3- <i>O</i> -galactoside, kaempferol 3- <i>O</i> -glucoside, myricetin 3- <i>O</i> -galactoside, myricetin 3- <i>O</i> -glucoside, quercetin, myricetin, kaempferol, luteolin/ gallic acid	leaves	Furlan 2004, Motta <i>et al.</i> 2005

Table 2.1. (Continued)

Species	Class of metabolite	Compound	Organ	Reference
<i>T. semidecantra</i>	tannin/ flavanol/ anthocyanin/ proanthocyanidin	1,2,6-tri- <i>O</i> -galloyl- β -D-glucose, 1,4,6-tri- <i>O</i> -galloyl- β -D-glucose, 1,2,3,6-tetra- <i>O</i> -galloyl- β -D-glucose, nobotanin A, B, C, D, E and F, casuarictin, pedunculagin, praecoxin A and B, casuarinin, 2,3- <i>O</i> -(<i>S</i>)-hexahydroxydiphenoyl-D-glucopyranose, castalagin, vescalagin, 1- <i>O</i> -methylvescalaginnobotanins A, B, F, 3,3'- <i>O</i> -dimethyl ellagic acid 4- <i>O</i> - α -L-rhamnopyranoside/ quercetin, myricetin, quercetin 3- <i>O</i> -(6"- <i>O</i> -galloyl) galactoside, quercetin 3- <i>O</i> - α -L-(2"- <i>O</i> -acetyl) arabinofuranoside, quercetin 3- <i>O</i> -arabnoside, quercetin 3- <i>O</i> -rhamnopyranoside/ malvidin 3-(<i>p</i> -coumaroylglucoside)-5-glucoside/ leucodelphinidin, leucocyanidin	aerial parts	Sirat <i>et al.</i> 2010, Yoshida <i>et al.</i> 1991, Harbone 1964, Lowry 1975
<i>T. urvilleana</i>	flavanol/ flavone/ anthocyanin/ triterpene	quercetin 3- <i>O</i> -arabnoside/ hispidulin 7- <i>O</i> - β -D-glucopyranoside / malvidin 3- <i>O</i> -(6- <i>O</i> - <i>p</i> -coumaryl- β -D-glucopyranoside)-5- <i>O</i> -(2- <i>O</i> -acetyl- β -D-xylopyranosyl)/ glutinol, taraxerol, α - and β -amyrin, β - sitosterol, ursolic and oleanolic acids	aerial parts	Perez-Castorena 2014, Terahara <i>et al.</i> 1993.

T. granulosa and *T. pulchra* have been characterized as possible biomonitors of air pollution, i.e. particulate matter and ozone (Klumpp *et al.* 2001, Furlan *et al.* 2004, Furlan *et al.* 2008, Santos & Furlan 2013, Zampieri *et al.* 2013, Espósito & Domingos 2014, Espósito & Domingos 2016, Pedroso *et al.* 2016). Moreover, as they occur mainly in open areas, such as forest edges and clearings, *Tibouchina* species are considered important for restoration/reforestation purposes (Ellison *et al.* 1993).

Tibouchina pulchra (Figure 2.1), popularly known as “manacá-da-serra”, has been increasingly used for urban ornamentation and, nowadays several cultivars are available in the flower market. The beauty and fascinating feature of these flowers is the development-associated color change: the buds open as white flowers that the next day become pink, further, from the third day towards senescent, the flowers maintain a deep pink color. The well-determined stages of flower coloring in *T. pulchra* raise interesting questions about the genetic and chemical mechanisms involved in the regulation of this phenomenon, which has up to now been unexplored (see Chapter 3).

In this context, this chapter presents results of isolation and identification of the petal tissue pigments of *Tibouchina pulchra*.

Figure 2.1. *Tibouchina pulchra* tree and its flowers.

2. MATERIAL AND METHODS

2.1. Plant Material

Pink petals of *Tibouchina pulchra* (cv. "manacá-anão") flowers were sampled at Praça Carlos José Gíglío (Latitude: -23.57998, Longitude: -46.73403) in the most vigorous flowering period (May and June/2016) between 8 and 9 a.m. Petals were immediately frozen in liquid nitrogen and stored at -80°C until processing. Samples were freeze dried in a lyophilizer (K202, Liobras) and crushed in a ball mill (Tissuelyser, Qiagen) for further analyses. An exsiccate was deposited on herbarium of Institute of Bioscience (SPF) of University of São Paulo (ID: Furlan73).

2.2. Extraction and analysis by UPLC-MS

Phenolic compounds were extracted from 100 mg of petal powder with two times of 1.5 mL of 0.2 % HCl in methanol (MeOH). The samples were sonicated for 10 min and centrifugated at 10,000 rpm for 10 min. The extract was filtered (PTFE 0.45 µm) and analyzed by Ultra Performance Liquid Chromatography (UPLC) system with Diode Array Detector (DAD) (Dionex Ultimate 3000) and Electrospray Ionization Quadrupole Time-of-Flight High-Resolution Mass Spectrometry (ESI-QTOF-HRMS) detector (Bruker Maxis), MS/MS analysis was performed with a Broadband Collision Induced Dissociation (bbCID) detector. Separation was achieved by using a C18 column (Waters Acquity UPLC 100 x 2.1 mm - 1.7 µm) at a flow rate of 0.3 mL min⁻¹, and 4 µL of injection volume, the column temperature was 45°C, and the solvent system composed of 1% formic acid in water (A) and 1% formic acid in acetonitrile (B). Gradient elution: 5 to 25% of B (0- 40 min), 25 to 100% of B (40- 42 min), 100% of B (42- 42.5 min), 100 to 5% of B (42.5- 43 min), 5% of B (43- 46 min). Separated compounds were first monitored using DAD (200 to 600 nm) and then MS scans were performed in positive ion mode (MS⁺), in the range m/z 75–1250 m/z, in the following conditions: capillary voltage set to 4,500 V, end plate offset at -500 V, nebulizer at 2 Bar, dry gas 12 L min⁻¹ and dry gas temperature at 200°C MS was calibrated using sodium formate. All data were processed using Data analysis software 4.2 (Bruker). This analysis was performed in the Department of Chemistry at Denmark Technical University under the supervision of Ph.D. Mads H. Clausen.

2.3. Isolation by preparative HPLC and identification by Nuclear Magnetic Resonance (NMR)

Also in partnership with Ph.D. Mads H. Clausen, flavonoids were isolated from 10 g of petal powder by extracting four times in 200 mL of hydrochloric acid 0.2% in MeOH. The samples were sonicated for 15 min, pillowed for 10 min, vacuum filtered and concentrated with a rotary evaporator. The crude extract was diluted to, approximately, 250 mg/mL and injected in a preparative High Performance Liquid Chromatography (HPLC- Waters) with Photodiode Array Detector (PAD). Separation was achieved on a C18 column (Waters X Bridge BEH ODB 30 x 150 mm, 5µm) at a flow rate of 20 mL min⁻¹, using 1 mL of injection volume, and a solvent system composed of 1% formic acid in water (A) and 1% formic acid in acetonitrile (B). Gradient elution: 10% of B (0- 3 min), 10 to 15% of B (3- 30 min), 15% of B (30- 50 min), 15 to 20% of B (50- 60 min), 20 to 25% of B (60- 80 min), 25 to 35% of B (80- 90 min), 35 to 45% of B (90- 95 min), 45 to 100% of B (95- 96 min), 100% of B (96- 98 min), 100 to 10% of B (98- 98.5 min), 10% of B (98.5- 102 min), and monitored using DAD (200 to 600 nm). Fractions were collected by time. All the fractions were concentrated by rotary evaporator. An aliquot was resuspended in 0.2% HCl in MeOH to check the purity by UPLC-MS. For the isolated compounds the dried sample was dissolved in deuterated dimethylsulfoxide (DMSO-d₆) for NMR analysis. ¹H and ¹³C NMR spectra were obtained by using a AVANCE III HD spectrometer (Bruker) operating at frequency of 800 MHz and equipped with a 5 mm TCI CryoProbe (Bruker). Two-dimensional analysis (HSQC and HMBC) were also performed. All data were processed using MestreNova software 11.0 (Mestrelab Research). The identification of compounds was performed in collaboration with Ph.D. Marcelo José Pena Ferreira from Department of Botany, University of São Paulo.

3. CONCLUSION

Melastomataceae and, in particular, *Tibouchina* are chemically poorly characterized taxons. Here, out of the sixteen compounds described for the first time in *T. pulchra*, five of them described for the first time in Melastomataceae, moreover an unpublished compound was identified as kaempferol 4'-O-(2''-methyl)- α -D-glucopyranoside. The advances in spectrometric techniques offer a unique opportunity to improve our knowledge about the chemical structure of natural products. Studies about flower anthocyanins are scarce and the understanding of their structure, biosynthesis and the regulatory mechanisms involved in their accumulation pattern can improve our knowledge about plant secondary metabolism, the relationship between flower and pollinators, and bring new insights for future biotechnological applications.

4. REFERENCES

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Chapter 3 – The regulation of floral color change in *Tibouchina pulchra*.

1. INTRODUCTION

Tibouchina pulchra (Cham.) Cogn. (Melastomataceae, Myrtales), named as “manacá da serra”, is a native species from the Atlantic Forest. Its use for urban ornamentation is increasing due to its beautiful flowers. In this sense, the ornamental dwarf cultivar of *T. pulchra* (“manacá-anão”) blooms with the same temporal pattern of floral color as the wild parental: buds, first stage (S1), open as white flowers (S2) that, one day after, become light pink (S3), further, flowers are deep pink (S4), from the third day towards senescence (Figure 3.1). As reviewed in chapter 1, the temporal alteration of flower color can be regulated by pollination, gene expression regulation, the presence of copigments and flavonoid-metal complexation, resulting in the differential accumulation of phenolic acids, flavonols, and anthocyanins extensively characterized in chapter 2.

Figure 3.1. Stages of floral color change of *T. pulchra*. S1- buds (day 0), S2- white flowers (day 1), S3- light pink (day 2), S4- dark pink (day 3).

T. pulchra flowers are nectarless, with poricidal anthers, hermaphrodite, heterantherous, herkogamous and self-compatible (Renner 1989, Brito & Sazima 2012). Concerning pollinators, Pereira *et al.* (2011) observed fifteen species of floral visitors in *T. pulchra*, being only four described as effective pollinators (*Bombus morio*, *B. pauloensis*, *Xylocopa frontalis* and *X. brasiliatorum*). These large bees interact with poricidal anthers transferring the pollen to the stigmas by vibration. *Xylocopa* species collected pollen exclusively from white flowers, while *Bombus* visited pink flowers only at the end of the flowering season, when white flowers are scarce. Moreover, by performing controlled pollination experiments, it was demonstrated that the pollen viability, stigma receptivity and fruit setting were similar in white and pink flowers. This data together with the bee color preference described above suggest that flower color change is not associated to pollination but, according to Da Silva (2006), may enlarge the visitor diversity.

As aforementioned, the pigments responsible for the color of *T. pulchra* flowers are phenolic acids and flavonoids (flavonols and anthocyanins). These phenolic compounds are produced by the combination of products from shikimic (SKM) and malonate (MAL) pathways, whose building blocks are derived from primary metabolism (Figure 3.2). Many simple benzoic acid derivatives (*e.g.* gallic acid, C₆C₁) and phenylpropanoids (*e.g.* *p*-coumaric, C₆C₃) are produced by SKM pathway, while flavonoids (C₆C₃C₆) are biosynthesized by intermediates from both, SKM and MAL pathways (Davies *et al.* 2009).

PHENYLALANINE AMMONIA LYASE (PAL) is the first enzyme in the phenylpropanoid and flavonoid pathway, it catalyzes the nonoxidative deamination of phenylalanine yielding *trans*-cinnamic acid. CINNAMATE 4-HYDROXYLASE (C4H) is a CYTOCHROME P450 MONOOXYGENASE (P450) enzyme involved in the hydroxylation of *trans*-cinnamic acid at C4 position to produce *p*-coumaric acid. This is a branching point that can channel the metabolic flux towards phenylpropanoids or flavonoids. By the action of COUMARATE 3-HYDROXYLASE

(C3H), cinnamic acid derivatives as caffeic, ferulic, and sinapic acids are synthesized. On the other hand, *p*-coumaric acid is activated to the corresponding Co-A thioester facilitating further conversion to flavonoids, this reaction is catalyzed by 4-COUMARATE:CO-A LIGASE (4CL) (Dewick 2009, Saito *et al.* 2013).

The first produced flavonoid class comprises the chalcones, which are formed by the action of CHALCONE SYNTHASE (CHS). This enzyme belongs to a family of type III polyketide synthases and is the first committed enzyme in biosynthesis of flavonoids. Three sequential additions of the extender molecule malonyl-CoA in a *p*-coumaroyl-CoA is the reaction catalyzed by CHS (Dewick 2009, Saito *et al.* 2013). Chalcones are the first colored flavonoid, and provide yellow coloration to petals of a few plant species (*e.g.* *Dianthus caryophyllus* L., Caryophyllaceae, Caryophyllales) (Ono *et al.* 2006a, 2006b). CHALCONE ISOMERASE (CHI) acts in the stereospecific cyclization of naringenin chalcone to flavanone. FLAVANONE 3-HYDROXYLASE (F3H) is responsible for the oxygenation at C3 position of flavanone to form dihydroflavonol (dihydrokaempferol). FLAVONOID 3'-HYDROXYLASE (F3'H) and FLAVONOID 3'-5'-HYDROXYLASE (F3'5'H) are P450 that catalyze the hydroxylation at C3' and C3'-C5'-positions of the B-ring of flavonoid, respectively, rendering dihydroflavonols.

FLAVONOL SYNTHASE (FLS) and DIHYDROFLAVONOL REDUCTASE (DFR) are competitors for the same substrate, dihydroflavonol, a branching point for flavonols and anthocyanins formation (Pelletier *et al.* 1997). FLS catalyzes the double bond formation between C2 and C3, while DFR reduces the C4-keto group of dihydroflavonol to the corresponding leucoanthocyanidin. FLS, F3H and ANTHOCYANIDIN SYNTHASE (ANS) are the three 2-OXOGLUTARATE/FE(II)-DEPENDENT DIOXYGENASE (2-ODD) enzymes in the flavonoid biosynthesis pathway. The enzyme ANS catalyzes the formation of anthocyanidin from leucoanthocyanidin. Anthocyanidins exhibit a conjugated double bond system encompassing A, B and C rings, the later with two double bonds carrying a positive charge. Anthocyanidins are the chromophore moiety of anthocyanins, which are formed after glycosylation, acylation and/or methylation by the action of specific enzymes as FLAVONOID GLUCOSYLTRANSFERASE (FGT), ANTHOCYANIDIN ACYLTRANSFERASE (ACT) and/or O-METHYLTRANSFERASE (OMT) (Figure 3.2). In *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae, Brassicales), FGT can transfer glucose to the C3 position of both, flavonol and anthocyanin aglycones (Yonekura *et al.* 2012).

Due to anthocyanins appeal as natural food colorant, nutraceutical and bioactive compounds (Norberto *et al.* 2013, De Ferrars *et al.* 2014, Xiaonan *et al.* 2016, Gowd *et al.* 2017),

the regulation of anthocyanin biosynthetic pathway has been extensively explored. Most of the structural genes have been isolated (Winkel-Shirley 2001) and several regulatory genes have been characterized. *Petunia* (*Petunia hybrid* Vilm., Solanaceae, Solanales) and snapdragon (*Antirrhinum majus* L., Plantaginaceae, Lamiales) are the major model species for the study of anthocyanin biosynthesis, especially in floral tissues, however, maize (*Zea mays* L., Poaceae, Poales) and *Arabidopsis thaliana* have been extensively studied as well (Cone *et al.* 1986, Goodrich *et al.* 1992, Quattrocchio *et al.* 1998, 1999, Broun 2005, Dixon *et al.* 2005, Koes *et al.* 2005, Grotewold 2006).

Regarding the position in the pathway, structural genes can be grouped into two classes: early biosynthetic genes (e.g. *PAL*, *C4H*, *CHS*, and *FLS*) and late biosynthetic genes (e.g. *DFR* and *ANS*) (Tornielli *et al.* 2009). The early genes of anthocyanin pathway were functionally characterized in many plant species such as *A. thaliana*, *Solanum lycopersicum* L. (Solanaceae, Solanales), *Vitis vinifera* L. (Vitaceae, Vitales), *P. hybrida*, and *A. majus* (Sparvolli *et al.* 1994, Brugliera *et al.* 1999, Mol *et al.* 1983, Guo *et al.* 2009, Huang *et al.* 2010), while late genes (*DFR* and *ANS*) have been characterized mostly in *P. hybrida* and *A. thaliana*. (Pelletier *et al.* 1997, Devic *et al.* 1999). However, much less is known about the subsequent modifications (substituents), such as glycosylation, acylation, and methylation (Grotewold 2006). In fact, there are several types of substituents that guarantee the chemical diversity in flower color. For example, *P. hybrida* synthesizes methylated anthocyanins with an acylated rutinose moiety, while *A. thaliana* accumulates more complex anthocyanins with seven sugar and acyl moieties but no methyl groups (Brouillard *et al.* 2003, Saito *et al.* 2013).

Figure 3.2. Simplified schematic representation of flavonoid biosynthetic pathway. Primary metabolism is highlighted in green. Abbreviations indicate the following enzymes: 4-COUMARATE:CO-A LIGASE (4CL), ANTHOCYANIDIN ACYL TRANSFERASE (AACT), **ANTHOCYANIDIN SYNTHASE (ANS)**, CAFFEIC ACID O-METHYLTRANSFERASE (COMT), **CINNAMATE 4-HYDROXYLASE (C4H)**, CHALCONE ISOMERASE (CHI), CHALCONE REDUCTASE (CHR), **CHALCONE SYNTHASE (CHS)**, Coenzyme A (CoA), COUMARATE 3-HYDROXYLASE (C3H), DIHYDROFLAVONOL 4-REDUCTASE (DFR), erythrose 4-phosphate (E4P), FLAVANONE 3-HYDROXYLASE (F3H), FLAVONE SYNTHASE (FS), FLAVONOID 3'-HYDROXYLASE (F3'H), FLAVONOID 3'5'-HYDROXYLASE (F3'5'H), FLAVONOID GLYCOSYL TRANSFERASE (FGT), **FLAVONOL SYNTHASE (FLS)**, HSCoA: coenzyme A, ISOFLAVONE SYNTHASE (IFS), malonate (MAL), O-METHYLTRANSFERASE (OMT), **PHENYLALANINE AMMONIA LYASE (PAL)**, phosphoenolpyruvate (PEP), SHIKIMATE DEHYDROGENASE (SDH), and shikimate (SKM). The enzymes studied in the present work are highlighted in bold. Dotted lines represent more than one enzymatic reaction. Adapted from Dewick *et al.* (2009).

Numerous studies have demonstrated the transcriptional regulation of anthocyanin biosynthesis. A transcription factor complex (MBW complex) composed by a MYELOBLASTOSIS (MYB), a BASIC HELIX-LOOP-HELIX (bHLH), and a BETA-TRANSDUCIN REPEAT (WD40) protein, has shown to regulate the expression of the structural genes (Koes *et al.* 2005, Hichri *et al.* 2011, Xu *et al.* 2014, 2015). In vegetative tissues of *A. thaliana*, an eudicot, anthocyanin early biosynthetic genes are co-activated by bHLH and WD40 transcription factors in a MYB-independent manner, while the late biosynthetic genes require all three proteins to form the active MBW complex. Interestingly, in maize, a monocot, early and late biosynthetic genes are transcriptionally activated exclusively by MYB and bHLH proteins (Petroni & Tonelli 2011). By the characterization of mutant genotypes and transgenic lines, the transcriptional induction of anthocyanin biosynthesis in flowers has been addressed in several species, where distinct combinations of the aforementioned transcriptional factors have shown to be needed to determine floral pigmentation. MYB transcription factors induce early and late biosynthetic genes in *Nicotiana tabacum* L. (Solanaceae, Solanales), *Brassica oleracea* L. (Brassicaceae, Brassicales), *Anthurium andraeanum* Linden ex André (Araceae, Alismatales), *Mimulus lewisii* Pursh (Scrophulariaceae, Lamiales), and *M. cardinalis* Douglas ex Benth. flowers (Chiu *et al.* 2010, Pattanaik *et al.* 2010, Li *et al.* 2016, Yuan *et al.* 2016). In *Ipomea purpurea* (L.) Roth (Convolvulaceae, Solanales) transposon-insertion double mutants for *bHLH* and *MYB* genes resulted in snow-white flowers where the expression of the late biosynthetic genes was completely abolished (Park *et al.* 2007).

Additionally, the existence of post-transcriptional regulatory mechanisms has also been verified to control anthocyanin biosynthesis. In *A. thaliana*, it has been reported that the expression of the anthocyanin-associated MYB transcription factors is regulated by miRNAs and tasiRNAs-mediated hetero-silencing (Rajagopalan *et al.* 2006, Hsieh *et al.* 2009). In particular, the miR156 targets SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) mRNA, whose product destabilizes the MBW transcriptional activation complex. Thus, increased miR156 activity promotes the accumulation of anthocyanins by the MBW complex-mediated transcriptional induction of the late biosynthetic genes (Gou *et al.* 2011). Moreover, in *Petunia*, *CHS* has also shown to be post-transcriptionally regulated by siRNA (Koseki *et al.* 2005). Interestingly, anthocyanin accumulation in maize kernels is controlled by the regulation of the splicing pattern of *Bz2* gene, a GLUTATHIONE S-TRANSFERASE (GST) that mediates transfer of cytoplasmic anthocyanin into the vacuole (Pairoba & Walbot 2003).

Finally, post-translational regulation has also been reported for anthocyanin biosynthesis. In *A. thaliana*, KELCH REPEAT F-BOX proteins have shown to physically interact with four PAL isozymes inducing their 26S proteasome-mediated ubiquitination, resulting in the downregulation of phenylpropanoid biosynthesis (Zhang *et al.* 2013).

Although for some biological systems the regulation of anthocyanin biosynthesis has been elucidated, including the identification of master transcription factors (Butelli *et al.* 2008), many gaps remain unsolved for the complete understanding of the mechanisms underneath the accumulation of these pigments. Improving the knowledge about the multilevel regulation of gene expression will favor the development of new biotechnological tools for the generation of new varieties with enhanced health-promoting properties and visual appeal (Hichri 2011).

Some studies showed that metals can influence the color by establishing a flavonoid-metal complex. In cornflowers (*Centaurea sp.*, Asteraceae, Asterales), the bright blue color is the result of a supramolecular structure with stacking between copigments, pigments and metals: six molecules of apigenin, six molecules of cyanidin and four metal ions (Fe^{3+} , Mg^{2+} and two Ca^{2+}) (Shiono *et al.* 2005). Hortensia flowers (*Hydrangea macrophylla* (Thumb.) Ser., Hydrangeaceae, Cornales) are the best-known example of flavonoid-metal complexation influencing the corolla color. Under high aluminum content and acidity of the soil, the flowers are blue due to a nonstoichiometric complex with Al^{3+} , delphinidin 3-glucosides, quinic acid derivatives, while upon low aluminum concentrations, the same metal-pigment-copigment complex became light pink, as the result of soil pH change (Yoshida *et al.* 2003, Kondo *et al.* 2005, Oyama *et al.* 2015, Trouillas *et al.* 2016).

The pH affects the final color of flower petals by determining the conformation and absorption spectrum of anthocyanins. The light blue petals of morning glory (*Ipomoea tricolor* Cav., Convolvulaceae, Solanales) owe their color to the effect of the alkaline pH on the accumulated anthocyanins. The closed buds of these flowers are purplish red and their cells have a pH of 6.6. However, when the flowers open, the petal cell pH increases up to 7.7, and the pigment changes color to sky blue (Yoshida *et al.* 1995). Recently, studying the genetic determinants of *P. hybrida* flower pigmentation, Verweij *et al.* (2016) have identified PH3 protein, a WRKY-type transcriptional factor that regulates the expression of the proteins responsible for the vacuolar acidification.

The well-determined stages of flower coloring in *T. pulchra*, raise interesting questions about the genetic and chemical mechanisms involved in the regulation of this phenomenon,

which has been unexplored up to now. Here, we addressed this issue by cloning key flavonoid biosynthetic genes and profiling mRNA levels, pigment composition and metal content along flower development.

2. MATERIAL AND METHODS

2.1. Plant material

Petals at S1 to S4 stages (Figure 3.1) were sampled from five *Tibouchina pulchra* (cv. “manacá-anão”) plants located in Praça Carlos José Gíglio (Latitude: -23.57998, Longitude: -46.73403) in the most vigorous flowering period (May and June/2016) between 8 and 9 a.m. For each developmental stage a pool of petals (~ 5 g) from each plant was considered a biological replicate (n = 5). Samples were collected with gloves, in a sterile tube and immediately frozen in liquid nitrogen. Petals were stored at -80°C for mRNA extraction, or freeze dried in a lyophilizer (K202, Liobras) for chemical analysis. All material was crushed in a ball mill (TissueLyser, Qiagen) for further analyses. For gene cloning a pool of all stages collected in May and June/2014 was used. An exsiccate was deposited on herbarium of Institute of Bioscience (SPF) of University of São Paulo (ID: Furlan73).

2.2. Pigment profile

Phenolic profile was assessed analyzing petal extracts by Ultra Performance Liquid Chromatography (UPLC) system with Diode Array Detector (DAD) (Dionex Ultimate 3000) and Electrospray Ionization Quadrupole Time-of-Flight High-Resolution Mass Spectrometry (ESI-QTOF-HRMS) detector (Bruker Maxis). The extraction was performed as described in chapter 2 (item 2.2). To quantify the compounds, the areas of MS⁺ chromatograms were compared with standard curves of *p*-coumaric acid, kaempferol and cyanidin. This analysis was performed in the Department of Chemistry at Denmark Technical University under the supervision of Ph.D. Mads H. Clausen.

2.3. Gene cloning and expression

2.3.1. Sequence analysis for primers design

The sequences of the functionally characterized genes *PAL* (AT2G37040.1), *C4H* (AT2G30490.1), *CHS* (AT5G13930.1), *FLS* (AT5G08640.1) and *ANS* (AT4G22880.1) from *Arabidopsis thaliana* (Wanner *et al.* 1995, Bell-Lelong *et al.* 1997, Pelletier *et al.* 1997, Feinbaum & Ausubel 1998) were used as query to identified, by tBLASTx program (Altschul *et al.* 1997), the orthologous sequences from *Vitis vinifera*, *Eucalyptus grandis* W.Hill (Myrtaceae, Myrtales), and *Solanum lycopersicum* fully sequenced genomes available in the Phytozome v 9.0 database

(<http://www.phytozome.net/>). The amino acid coding sequences were aligned using the MUSCLE program following the standard configurations of the MEGA 6 package (Tamura *et al.* 2011). After the identification of conserved domains, the alignments were converted to nucleotides for degenerated primer design based on *Eucalyptus grandis* sequences, which is the *T. pulchra* closest related species with completely sequenced genome (Supplemental Figure 3.1 and Supplemental Table 3.2). The quality of the designed primers was verified using the software Oligo Analyzer 3.1 (<http://www.idtdna.com>).

Additionally, using the same strategy described above, primers were designed for the cloning of two reference genes for Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) normalization. The chosen genes were those previously described for gene expression analyses in *Petunia hybrida* flowers, *ELONGATION FACTOR 1 α* (*EF1*) and *RIBOSOMAL PROTEIN S13* (*RPS*) (Mallona *et al.* 2010).

2.3.2. RNA extraction and cDNA synthesis

Total RNA from 3 g of petal samples was extracted with CTAB (Cetyl trimethylammonium bromide- Chang *et al.* 1993) (Supplemental Figure 3.2). DNA was removed with 100 U of amplification-grade DNase (Invitrogen) following the recommended protocol. cDNA was synthesized from 1 μ g of RNA using oligo dT (for gene cloning) or random primers (for RT-qPCR) and the SuperScript III kit (Invitrogen). cDNA quality was confirmed by PCR using *RPS* constitutive gene primers.

2.3.3. Cloning

Gene fragments were amplified by PCR in a total volume of 50 μ L containing 0.2 mM each dNTPs, 0.2 μ M each primer (Supplemental Table 3.2), 1X reaction buffer, 1.5 mM MgCl₂, 50 ng of cDNA, and 2 U of Taq DNA polymerase (Invitrogen). The amplification conditions were: 94°C for 3 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and then 72°C for 10 min. Amplification products were purified (Kit GFX Amersham Biosciences) and cloned into a TOPO-TA vector (Dual promoter Kit Invitrogen) following the recommended protocols. Transformation was carried by using 50 μ L of *Escherichia coli* DH10B competent cells with 2 μ L of the ligation product. The mixture was kept on ice for 30 min, and subjected to a thermal shock of 45 sec at 65°C and 2 min on ice. 500 μ L of SOC (Super Optimal Broth) medium (Hanahan 1983) was added for a shaking incubation (45 min at 37°C). Samples were centrifuged (3 min, 3,000 rpm at room temperature) and cells resuspended and plated on Luria-Bertani (LB)

agar (Bertani *et al.* 1951) with kanamycin, X-Gal (5-bromo-4-chloro-3-indoxyl- β -D-galactopyranoside) and IPTG (isopropil β -D-1-thiogalactopiranosida). Five positive colonies for each gene were grown in liquid LB and plastidial DNA was purified and sequenced with vector universal primers.

2.3.4. Sequence analyses

To verify the identity of the cloned *T. pulchra* gene fragments, phenetic analyses were performed. Orthologous sequences from *Arabidopsis thaliana* (AT) and *Brassica rapa* L. (Brara)-Brassicaceae, Brassicales, *Eucalyptus grandis* (Eucgr)- Myrtaceae, Myrtales, *Mendicago trunculata* (Medtr) and *Trifolium pratense* L. (Tp) – Fabaceae, Fabales, and *Solanum lycopersicum* (Soly) – Solanaceae, Solanales, were retrieved from Phytozome v 12.1 database (<http://www.phytozome.net/>) by tBLASTx program (Altschul *et al.* 1997). The species were selected according to the following criteria: i) fully sequenced genome, ii) genes belonging to gene families whose functional annotation was associated to phenolic compound biosynthesis (gene family 94476114 for *PAL*, 94469571 for *C4H*, 94475332 for *CHS*, 94475392 for *FLS*, 94470119 for *ANS*, 94475526 for *RPS* and 94476231 for *EF1*), and iii) species placed in Rosids clade in APG IV classification (AT, Brara, Eucgr, Medtr and Tp, and Soly as outgroup). The coding sequences were aligned using the Clustal program following the standard configurations of the MEGA 6 package (Tamura *et al.* 2011), with manual verification of each codon according to the amino acid sequence. The phenograms were constructed with the following parameters: *Neighbor-joining*, *Bootstrap* of 1,000 replicates and the best model test for each analysis.

2.3.5. RT-qPCR

Based on *T. pulchra* sequences, gene specific primers for expression analysis were designed to amplify fragments of approximately 160 bp (Supplemental Table 3.2). To quantify mRNA levels by RT-qPCR, reactions were carried out in a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems) using 2X Power SYBR Green Master Mix reagent (Life Technologies) in 14 μ L final volume. Absolute fluorescence data were analyzed using the LinRegPCR software package (Ruijter *et al.* 2009) to obtain quantitation cycle (Cq) values and calculate primer efficiencies (the primer efficiency values ranged from 1.85 to 1.98). Expression values were normalized against the geometric mean of the two reference genes (*EF1* and *RPS*), according to Quadrana *et al.* (2013). A permutation test lacking sample distribution assumption

(Pfaffl *et al.* 2002) was applied (formula below) to detect statistical differences ($P < 0.05$) in expression ratios using the algorithms in the fgStatistics software package (Di Rienzo 2009).

$$RE = \frac{Pe_{goi}^{\wedge (\text{control Ct mean} - \text{sample Ct mean})}}{Pe_{ref}^{\wedge (\text{control Ct mean} - \text{sample Ct mean})}}$$

RE: relative expression, Pe: primer efficiency, goi: gene of interest, Ct: threshold cycle, control Ct: Ct mean of control biological replicates, sample Ct: Ct mean of sample biological replicates, ref: reference gene.

2.4. Metal content

To analyze the content of metals 500 mg of freeze-dried powder petals were sent to Laboratory of Vegetal Tissue (ESALQ, USP). After a nitro-perchloric digestion, contents of Mg, K, Na, Ca, Cu, Mn, Zn and Fe^{3+} were determined by Atomic Absorption Spectroscopy (AAS).

2.5. Data analyses

Statistical analysis for pigment and metal profiles were performed using Infostat package (Di Rienzo *et al.* 2011). When the data set showed normality, ANOVA followed by Tukey test ($P < 0.05$) were performed to compare differences between groups. In the absence of normality, a non-parametric comparison was performed by applying the Kruskal-Wallis test ($P < 0.05$). All values represent the mean of five biological replicates.

3. CONCLUSION

In this work, by applying a combined approach of detailed biochemical and molecular genetic techniques, we characterized the pigment profile and investigated the mechanism that determines *T. pulchra* flower color change. The thirty phenolic compounds identified in chapter 2 were quantified showing variations in pigment and copigment contents along flower development. The partial cloning of five key genes of flavonoid biosynthetic pathway allowed to profile mRNA levels that explained, at least in part, the pigment accumulation pattern. Collectively these data indicate that the flower color change in *T. pulchra* is regulated by the transcriptional control of the structural genes of the flavonoid biosynthetic pathway that determine the accumulation of petunidin and malvidin, as well as, the copigment kaempferol *p*-coumaroylhexoside. Additionally, the quantification of metal contents suggested that Fe³⁺ might influence the saturation of the color at dark pink stage.

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