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MOLECULAR CHARACTERIZATION OF GENES INVOLVED IN THE PRESENCE OF ANTHOCYANINS IN EGGPLANT (*S. melongena* L.)

SSD AGR/04 Orticoltura e Floricoltura

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Summary

Abstract

Eggplant (*Solanum melongena* L.) is a berry-producing vegetable belonging to the large *Solanaceae* family. Eggplant berries are rich in important secondary metabolites like phenolic compounds (chlorogenic acid in the flesh and anthocyanins in the peel) and flavonols, two classes of antioxidant molecules with suggested health-promoting effects in humans. Anthocyanins pathway is one of most investigated and well characterized pathway among the higher plants. However, the information available for eggplant anthocyanins pathway are rather limited whit the respect to other Solanaceous crop like tomato (*S. lycopersicum*) and potato (S. *tuberosum*). As part of this Ph.D. project, focusing on the highly conserved MYB-bHLH-WD (MBW) transcriptional complex model, which is pivotal in the transcriptional regulation of many anthocyanins related genes, the eggplant MBW were identified and functional characterized. Moreover, a regulatory R3 MYB type repressor (*Smel-MYBL1*), never reported before, was identified and characterized as well. Fruit peel pigmentation of different varieties of eggplant can vary widely from white to green to purple and violet; in particular, the black/dark purple and dark/light violet peel pigmentations reflect the alternative presence of two different anthocyanins: delfinidine-3-rutinoside (D3R) and delphinidin-3-[p-coumaroylrutinoside]-5-glucoside (nasunin), respectively. The conversion of D3R into nasunin is still an unelucidated part of the anthocyanin pathway in eggplant. The genes responsible of this metabolic step, the *SmAAT* was identified and functionally characterized through complementation in genotypes carrying the recessive mutant *aat* allele. Moreover, another genes, *Sm5GT1*, putatively involved in this step were identified, but more efforts are needed to characterize it. Furthermore, the ripening process is a pivotal aspect which affects the anthocyanins content. The eggplant ripening is commonly divided into three stages: unripe (stage A), commercially ripen (stage B) and physiologically ripen (stage C). Anthocyanin pigments are accumulated to the highest levels at the unripe fruit stage and their levels dramatically decreased as ripening progresses (Mennella et al., 2012), although the total phenolic content remained quite stable (Esteban et al., 1992) leading to the hypothesis that some other still undetermined phenolics are produced during this stage instead of anthocyanins. Focusing on these evidences a list of phenols never reported before in the eggplant peel were identified by HPLC-DAD-UV-VIS analysis. The identification of those new compounds and the recent availability of eggplant genetic resources have allowed the identification of the genes putatively involved in the ripening metabolism of the eggplant berries. The characterization by RT-qPCR three genes were carried out: *SmFLS* and a couple of *SmGT*. This genes are now the best candidate for future experiments aimed at irrefutably proving this function.

Introduction

Eggplant

Eggplant, or aubergine, (*Solanum melongena* L.) is the second most economical important *solanaceous* fruit crop after tomato. It is member of the large and species-rich genus *Solanum* L. (*Solanaceae*), a large plant family comprising over 3000 species including many other important crops such as tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), pepper (*Capsicum annuum*) and tobacco (gen. *Nicotiana*). Indeed *S. melongena* L. is also known as "brinjal" in Asian countries where is one of the most produced and consumed vegetables. The importance of this crop has recently raised in view of the growing interest on the nutraceutical values of foods. Eggplant berries are good source of vitamins and minerals, and are particularly rich in iron (Grubben, 1977). Eggplant berries are also rich in important secondary metabolites and a good a source of antioxidants (Cao et al., 1996), in particular flavonoids and the phenolic chlorogenic acid (Stommell and Whitaker, 2003; Mennella et al., 2010). These compounds are present in both the fruit's flesh and peel (Huang et al., 2004) and their content and profile are developmentally regulated during fruit ripening (Mennella et al., 2012). The bitter taste is due to the presence of glycoalkaloids. In addition to its nutritional value, eggplant is known to have numerous beneficial qualities: fruit extracts have been shown to have anti-oxidant (Lo Scalzo et al., 2010), hepatoprotective (Akanitapichat et al., 2010), anti-carcinoma (Eleveld-Trancikova et al., 2005), anti-microbial, anti-LDL, anti-viral (Sudheesh et al., 1997; Matsubara et al., 2005) and cardio protective properties (Das et al., 2011).

Crop relevance

Eggplants global production was estimated in 2018 around 54.07 Mt with a turnover of around 0.2 billions of USD; in the last 10 years the harvested area and production (Fig. 1) from 1'600'000 Ha and 40 Million tons raised up to 1'850'000 Ha and 55 Million tons, respectively (FAO data 2018; http://www.faosta.fao.org). The production is mainly concentrated in Asia (93.6% of the world production), followed by Africa (3.84%) and Europe (1,79%). Mediterranean area represents an important spot for the eggplant cultivation (both in open field and greenhouse) and a secondary center of domestication: the top Mediterranean countries in term of productions are Egypt and Turkey, while among the EU countries Italy, Spain and Greece are the main producer. In Italy, the production lasts over the whole year in open field or in greenhouses and is locally marketed (fresh or processed) or is exported in northern EU countries. In 2018, the Italian production was estimated around 298'000 tonnes in a harvested area of 10'000 ha, predominantly concentrated in the southern regions (Sicily,

Campania, Puglia and Calabria), in which are located also most of the productions of berries destined specifically to processing (grilled and pickles).

Table 1: Top 10 countries in eggplant production (FAOSTAT data 2018)

Area	Production (Tonnes)	Area Harvested (ha)
China	34137557	804618
India	12826000	736000
Egypt	1409202	46849
Turkey	836284	23969
Indonesia	551552	44016
Japan	300400	8970
Italy	298313	9560
Philippines	244838	21651
Spain	238325	3619
Iraq	113699	6307
Algeria	181618	5978

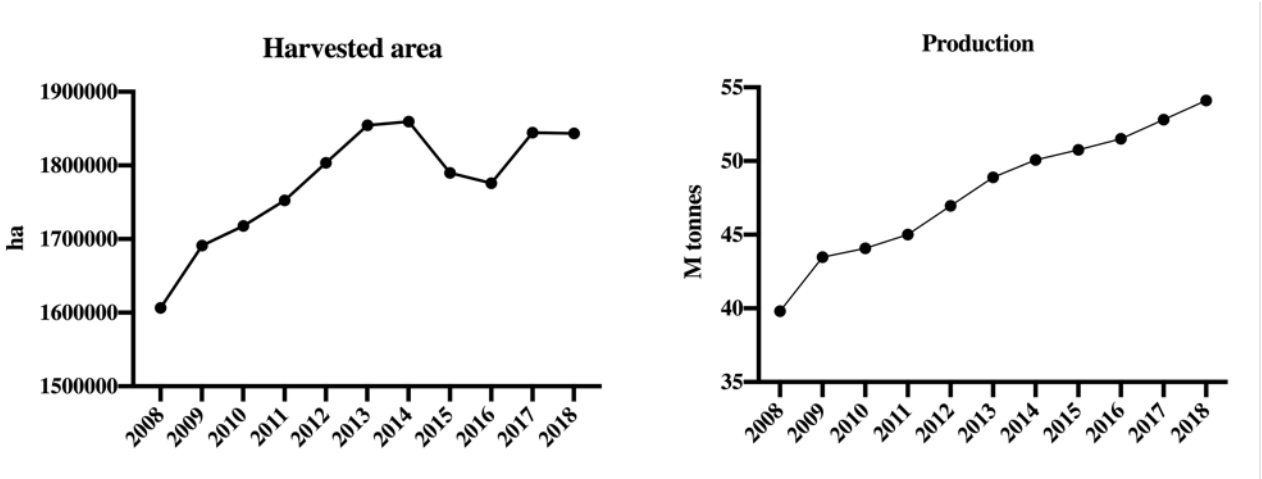

Figure1: trend of global harvested area and production in the decade 2008-2018 (source: Faostat)

Taxonomy and Botany

"Eggplant" term refers to three cultivated species: *S. melongena* L., *S. macrocarpon* L. and *S. aethiopicum* L.; *S. melongena* is the most common species and is cultivated worldwide, the last two species are indigenous to a vast area of Africa and are of minor economic importance, as they are mainly cultivated in some Central African countries. *S. aethiopicum*, called "melanzana rossa di Rotonda" is also grown in a restricted area in Southern Italy (Rotino et al., 2014). Usually, *S. melongena* and *S. macrocarpon* are included in section Melongena Dunal (Lester and Daunay, 2003;

Lester et al., 2011), while *S. aethiopicum* is assigned to the Oliganthes Bitter (Dunal) section. Taxonomically, all the three species are included in the *Solanales* order, *Solanaceae* family, *Solanoideae* subfamily, *Solaneae* tribe, *Solanum* genus, *Leptostemonum* subgenus. Members of *Solanum* subgenus *Leptostemonum* are defined by their sharp epidermal prickles and are commonly called "spiny solanums". The name *Leptostemonium* is due to another important characteristic of this subgenus: the presence of attenuate anthers with terminal small pores (Levin et al., 2006). The subgenus *Leptostemonum* includes around 450 currently recognized species worldwide distributed and mainly originated in the New World (Vorontsova and Knapp, 2012; Knapp et al., 2013). By contrast, the three cultivated eggplant species have been originated in Old World (Levin et al., 2006; Vorontsova and Knapp, 2016). *S. melongena* L. is indeed considered an Old World species that seems to be derived from the wild African species *Solanum incanum* L. and subsequently domesticated in India and southeast China (Daunay et al. 2001; Wang et al., 2008; Meyer et al., 2012; Cericola et al., 2013). In particular, during the eighth century, eggplant was spread from Asia, along the Silk Road, into Western Asia, Europe, and Africa by Arab, then it was introduced into America by Europeans (Prohens et al., 2005) and later worldwide spread. *Solanum melongena* named also common eggplant is characterized by a large morphological diversity, probably connected with the presence of different domestication centers. Vavilov, in 1951, considered *S. melongena* as native to the "Indo-Chinese center of origin". Recent archeological evidence suggests that utilization of wild eggplants may have started earlier in India than China, with another independent center of domestication in the Philippines (Meyer et al., 2012). The wild relatives of *S. melongena* are variable and intricate regarding to their taxonomic and phylogenetic relationships (Vorontsova et al., 2013). However, eggplant wild relatives have been classified based on their cross compatibility with the cultivated species into three genepools: the first one consists in cultivated *S. melongena* and its wild ancestor *S. incanum* (Fig 2) which can be crossed easily and produce normal fertile hybrids (Plazas et al., 2016; Ranil et al., 2017); the second genepool includes a large number of wild relatives like *S. dasyphyllum*, *S. linnaeanum* and *S. tomentosum* L. that can be crossed with eggplant, but the success of the crosses and the viability or fertility of the hybrids is low, and some derived interspecific hybrids are partially or completely sterile (Rotino et al., 2014; Kouassi et al., 2016). The third genepool includes many distantly related species like *S. torvum* Sw., *S. elaeagnifolium* Cav., and *S. sisymbriifolium* Lam., which breeders attempt to use for incorporating their resistance features into the eggplant gene pool, but seeds from sexual crosses are often hard to be obtained and any alternative strategies of the hybrid rescue have been tried to obtain interspecific hybrids (Kouassi et al., 2016; Plazas et al., 2016; Syfert et al., 2016).

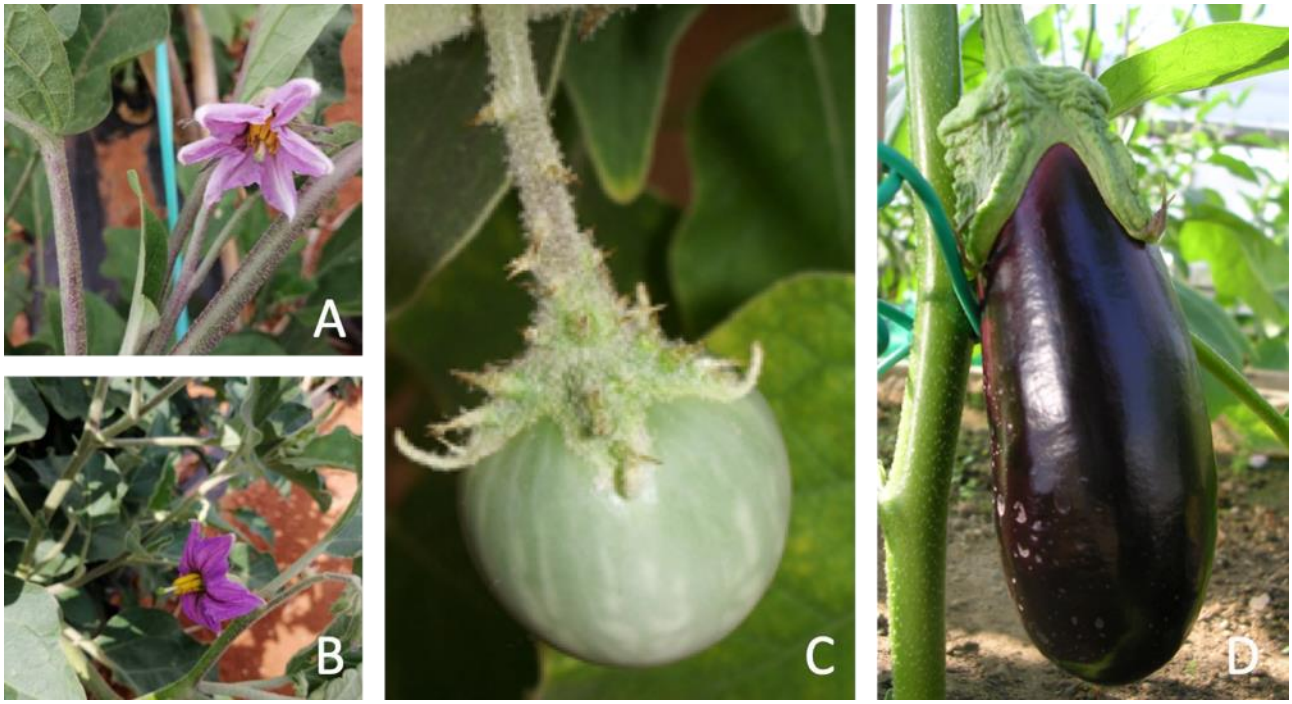

Figure 2. Representative flower and fruit morphology of eggplant and its wild relative. A) Solanum melongena L. flower, B) Solanum incanum flower, C) Solanum incanum fruit, D) Solanum melongena L. fruit.

Therefore, the knowledge of eggplant wild and allied relatives is very useful for the development of interspecific hybrids which may be used as sources of variation for breeding programs. Even if the relatives generally produce small, bitter, multi-seeded fruits, almost always inedible, and the plant are generally very spiny, some of them possess high levels of chlorogenic acid and other bioactive compounds, which may have potential interest for human health (Meyer et al., 2015). Moreover, new traits related to adaptation to climate change and also pest and disease resistance could be transferred to cultivated eggplant through the development of interspecific populations (Rotino et al., 2014). *Solanum melongena* L. plant is woody and develops several branches according to a roughly dichotomic ramification pattern. The stem is erect, initially herbaceous, then lignifies during the season, increasing its resistance to the wind and the weight of the fruits. Although some exceptions can be observed among cultivars, the height of the plant, when growing freely, generally varies between 60 and 150 centimeters; under protected cultivation where pruning is applied the height of the plants, grown with 2-3 stems, may be higher than 200-250 cm. Leaves are alternate, simple with a petiole ranging from 5 to 10 cm; leaf blead may be ovate and ovate-oblong, sized 3-25 x 2-15, cm long or more, leaf shape may be straight or dentate often without stipules. Different types of hairs are present on both sides of leaves, their density varies according the genotypes. Along the leaf veins, often are present prickles. The color of the leaves ranges from light green to dark green, but leaf pigmentations from light purple to dark violet are also common due to the presence of anthocyanins.

Trichomes and prickles may be present on all the vegetative tissues of the plants. Eggplant flowers are hermaphrodite, usually large 5-7 cm in diameter. The basic flower type is 5-merous (5 sepals, 5 petals, 5 stames) but 6,7, and 8-merous flowers are commonly found in globose and round fruited types. Petals may display different colors ranging from white to dark pink, purple or violet. the filaments are short and thick; anthers are bicameral, yellow and arranged around pistil. The ovary is superior with 2 or many celled, the style can be shorter (ipostyle), equal (mesostyle) or longer (longistyle) than stamens; stigma is, generally, green, headed and lobed. The maturation of the male and female organs generally occurs at the same time to favor self-pollination. Eggplant is prevalently autogamous but also the allogamy is possible due to anemophilous and entomophilous pollination facilitated by longistyled flower. Eggplant berries show, also as a result of the human selection, a wide variety of different typologies regarding fruit shape (round, oval and elongated), fruit dimension (from small berries of few grams to fruits reaching 1 kg and more), calyx coverage and presence of prickles on the calyx, peduncle length, firmness of flesh and hardness of skin. Based on the fruit shape, cultivars have been divided into three main botanical varieties: the round, oval or egg-shaped cultivars are grouped under *S. melongena* var. *esculentum* or common eggplant; the long slander types are included under *S. melongena* var. *serpentinum*, or snake eggplant; the small and straggling fruited plants belong to *S. melongena* var. *depressum* or dwarf eggplant (Martin, 1979). Typologies of eggplant berries also differ for their color, which ranges from a unique color (black, dark purple, dark violet, green, white) to a pigmentation characterized by a prevalent color combined with the presence of a secondary ones in form of stripes along the fruit or shades around and under the calyx. The anthocyanin present in the most common skin-colored cultivated varieties of eggplants is due to the accumulation in the peel of large amounts of two different typologies of anthocyanins: delfinidine-3-rutinoside (D3R) and delphinidin-3-[p-coumaroylrutinoside]-5-glucoside (nasunin), (Tigchelaar et al., 1968; Toppino et al., 2016). Azuma et al. 2008 showed how HPLC profiles dominated by nasunin are typical of ‘type 1’ (Japanese type) eggplants which present a lilac peel pigmentation, while ‘type 2’ accessions are characterized by the presence D3R and show a black/dark purple phenotype. Eggplant has been found to contain high contents of other phenolic compounds with antioxidant activity mostly represented by hydroxycinnamic acid conjugates (Cao et al., 1996; Stommel and Whitaker, 2003) ad chlorogenic acid in the flesh. Indeed, eggplant berries provide a relevant quantity of some minerals, like P, K, Ca or Mg (Flick et al., 1978; Savvas and Lenz, 1996). Regarding the mineral composition of fruits, the highest is K content (mean of 2366 mg kg^{-1}), followed by Na (382 mg kg^{-1}), Mg (257 mg kg^{-1}), P (221 mg kg^{-1}), Ca (170 mg kg^{-1}), and at much lower concentrations the Zn (1.88 mg kg^{-1}), Fe (1.34 mg kg^{-1}), and Cu (0.68 mg kg^{-1}) (Gisbert et al., 2011) (Table 2). Is well known that eggplant is highly regarded as a source of antioxidants (Cao

et al., 1996), in particular flavonoids and the phenolic chlorogenic acid (Stommell and Whitaker, 2003; Mennella et al., 2010). These compounds are present in both the fruit's flesh and skin (Huang et al., 2004) and their content and profile are developmentally regulated during fruit ripening (Mennella et al., 2012). Bitter taste is due to the presence of glycoalkaloids and saponins. In addition to its nutritional value, eggplant is known to have numerous beneficial qualities. Fruit extracts have been shown to have anti-oxidant (Lo Scalzo et al., 2010), hepatoprotective (Akanitapichat et al., 2010), anti-carcinoma (Eleveld-Trancikova et al., 2005), anti-microbial, anti-LDL; anti-viral (Sudheesh et al., 1997; Matsubara et al., 2005) and cardio-protective properties (Das et al., 2011).

Table 2: The composition of eggplant per 100 g of edible portion.

Compound	Quantity/100g	Compound	Quantity/100g
Calories	24.0 Kcal	Iron	0.24 mg
Water	92.41 g	Zinc	0.16 mg
Protein	1.01 g	Magnese	0.25 mg
Fat	0.19 g	Selenium	0.3 mg
Sodium	2.0 mg	Vit A	27 IU
Copper	0.17 mg	Vit B1	0.039 mg
Potassium	2.0 mg	Vit B2	0.037 mg
Sulphur	44.0 mg	Vit B3	0.649 mg
Chlorine	52.0 mg	Vit B5	0.281 mg
Fiber	1.3 g	Vit B6	0.084 mg
Oxalic acid	18.0 mg	Vit C	2.2 mg
Calcium	9.0 mg	Vit E	0.3 mg
Magnesium	14.0 mg	Vit K	3.5 mg
Phosphorus	25.0 mg	B-carotene	0.74 µg

Eggplant composition is affected by several factors, such as environmental conditions, growing systems, genetic background and ripening stage. Conversely to what happens in the other major Solanaceous fruit crops like tomato and pepper, in eggplant the commercial ripening stage and the physiological ripening stage are not coincident. The eggplant ripening is commonly divided in three stages: Stage A refers to immature fruits, Stage B represents the commercial ripening stage and is empirically determined, and Stage C is the physiological ripen stage (Mennella et al., 2012). Fruits at the Stage A and B show a pigmented and glossy peel, a firmer flesh containing immature seeds and a harder skin with respect to the Stage C, which on the contrary is characterized by the presence of mature seeds in the fruit, together with a loss of firmness in the flesh and a deep pigmentation change of the skin, which turns from the typical commercial colors to a wide range of browns, greys or yellows when fully physiologically ripe, together with a loss of glossiness and brightness. The change

of color and glossiness of the skin severely affects the commercial value of the fruits, which result unmarketable (Rotino et al, 1997). Consequently, the long stability of the berries' pigmentation along with the ripening and the fidelity to the right commercial typology are keys traits of interest considered in the breeding programs aimed at the improvement of the commercial value of fruit and to the new varietal constitution.

Cultivation and growth condition

Eggplant is traditionally grown in open field but also the greenhouse cultivation is common since the 60s in Europe. In tropical climates eggplant can be considered a short-lived perennial, while in temperate climates is grown as annual culture. The germination takes 15 days at the optima T° of 20-22°C, while at 12-13° C the germination lasts up to 4 weeks and can be reduced by up to 50%. Under 12° C the germination capacity is significantly reduced. The expansion of the cotyledons takes few days and the first true leaves appear after a week. The first flowers appear, depending on the variety, when 3-9 true leaves are developed. Every 2 leaves developed a new flower appears. Generally, the optimal growth temperature is between 15° C and 18° C at night, and between 25° C and 30° C during the day. Temperatures above 30-32° C are harmful for the plants as cellular respiration rate exceeds the photosynthetic process. At this temperature, leaves lose turgor, turn to yellow and then fall as well as fruits. The optimum relative air humidity ranges from 65 to 70 percentage points. Soil must be well drained to avoid water stagnation around the stem. On the other hands, a low water availability leads to the formation of low-quality fruits which grow stringy and bitter. The crop requires 340-515 mm of water per season. The soil pH must be between 5.5 and 6.8 (Panero, 1981). Grafting is mostly used in intensive cultural conditions. In Europe, eggplant was mostly grafted onto tomato or tomato interspecific hybrid (*L. esculentum* x *L. hirsutum*) which bring to the eggplant the resistance to many soil pathogens and the tolerance to low soil temperature (Ginoux and Laterrot, 1991). In the last decades, *Solanum torvum* has been widely employed because, beside resistance to soil borne diseases, increase both the vigor and yield of scion. Grafting on *Solanum integrifolium* as rootstock carries resistance to *Fusarium* and bacterial wilts (Yoshida et al 2004).

Breeding objectives

The main breeding objectives are focused on quantitative and qualitative aspects. Increasing yield and adaptation of cultivars to various growing conditions including adaptability to climate changes (temperate to tropical), delayed season of growth and production system (for open field or greenhouse cultivation) are fundamental goals (Daunay, 2001). Resistance to pathogens as well as tolerance to abiotic stresses are pivotal breeding objectives. Although high level of resistance to bacterial wilt

(*Ralstonia solanacearum*) and fruit anthracnosis (*Colletotrichum gleosporioides*) have been reported for some *S. melongena* cultivars, generally only low level of tolerance can be found against some of the most relevant diseases for this crop as Verticillium wilt (*Verticillium dahliae*), Fusarium wilt (*Fusarium oxysporum* f.sp. *melongenae*) and root knot nematodes (*Meloydogyne* spp.). On the contrary, wild relatives showed high level of resistance and tolerance both to diseases and some abiotic stress, as drought or salinity, which can be exploited as genetic sources in breeding programs (Daunay et al., 1991) employing breeding strategies either classical, if they are interfertile with *S. melongena*) or alternative if they are not (e.g embryo rescue, somatic hybridization) (Rotino et al., 2014). In the last decades the eggplant breeding goals have been mainly focused on the improvement of agronomic traits, including fruit size, weight, color, and shape (Aubert et al., 1989; Kashyap et al., 2003; Frary et al., 2006), reduced prickliness, yield potential and adaptation to climatic conditions (Daunay et al., 2001), resistance or tolerance to biotic stresses (Rotino et al., 2014). A long lasting of the commercial berries' pigmentation and a reduced number or absence of seeds (parthenocarpy) are important qualitative trait of commercial importance as well as the fidelity maintenance to the fruit typology when grown in different environments. Recently organoleptic and nutritional properties, bioactive and anti-nutritional compounds, post-harvest (Frary, 2006) and processing-related traits of eggplant fruits (flesh consistency and browning, absence of seeds) have been studied in a genetic/genomic perspective with particular attention to the chlorogenic acid and anthocyanin content (Prohens et al., 2007, 2013; Gajewski et al., 2009; Prohens et al., 2013; Plazas et al., 2013; Zhang et al., 2014; Docimo et al., 2016; Lo Scalzo et al., 2016). Regarding the plant, the more studied morphological traits are vigor, prickliness, height.

Eggplant genetic resources

Archaeological records suggest that eggplant was present in the diet of inhabitants of the Indus valley during Harappan civilisation, thus Rajasthan may have been an area of domestication (Kashyap et al. 2010). On the other hand, the use of eggplant as a vegetable crop was described in Chinese literature dating to 59 BCE (Wang et al. 2008). In turn, eggplant spread westwards to Persia and was introduced to the Mediterranean Basin by Arabs from 7th century (Daunay, 2008). Selection and breeding over some hundreds of years has resulted in the generation of a large number of heirloom and local eggplants. These are conventionally grouped as "Occidental" (preferred – grown in Mediterranean Basin, Europe and the Americas) and "Oriental" eggplants (East and Southeast Asia). They vary from one another both with respect to their overall plant morphology and physiology, fruit size, color and shape. Fruit color can be cream, green, red, reddish-purple, dark purple or black, and some varieties

produce striped fruits. Global trade is concentrated on an increasingly diminishing number of elite varieties (Ali et al., 2011). These include F1 hybrids (Daunay et al., 2008) which through their expression of heterosis for yield and their unique genetic status, have become extremely attractive for growers, seed suppliers and breeders (Rodriguez-Burrruezo et al. 2008; Munoz-Falcon et al. 2009). As a result of the spreading of commercial hybrids, the genetic diversity of materials in cultivation may be significantly reduced. The conservation and characterization of germplasm is therefore a priority, to preserve the genetic variation for future varietal improvement and for addressing future breeding challenges (Hurtado et al. 2012). Thousands accessions of eggplant are collected at research centers, universities and the major seed gene-banks distributed worldwide, and a number of investigations aiming to characterize the phenotypic and genetic diversity of local collections of eggplant have been published in recent years. The phenotypic and genetic diversity of local Spanish (Prohens et al. 2005; Muñoz-Falcón et al. 2011), Indian (Behera et al. 2006), Chinese (Ali et al. 2011) and Turkish (Demir et al., 2010) germplasm started to be studied. More recently, the morphological and molecular diversity among 52 accessions from three geographically well separated centres of diversity (China, Spain and Sri Lanka) has been assessed (Hurtado et al. 2012) while, thorough historic, morphologic and molecular data, Meyer et al. (2012) made assumptions on phylo-geographic relationships among candidate progenitors and Asian landraces. A collection of 238 eggplant breeding lines, heritage varieties and selections within local landraces provenanced from Asia and the Mediterranean Basin was collected at CREA-GB and genotyped using 24 microsatellite loci distributed uniformly throughout the genome (Cericola et al., 2013). This enabled the construction of a representative core collection of 191 accessions which was field-phenotyped for key breeding fruit and plant traits, and fingerprinted with 384 SNPs markers (Cericola et al., 2014). The phenotypic data were subjected to principal component and hierarchical principal component analyses, allowing three major morphological groups to be identified (Figure 3).

Figure 3 Fruits belonging to the three different morphological groups. Group 1: A) AM 269 – Talindo, B) AM 026 – Dr2; Group 2: C) AM 168 – angiò, D) AM 031 – FantE63D, E) AM 160 Dourga; group 3: F) AM 037 Violetta di Toscana, G) AM 291 17 CAAs, H) AM 210 – 67/3.

Population structure and descriptions of linkage disequilibrium characteristics in eggplant was established. Associations with several phenotypic trait were assessed (Portis et al., 2014). Finally, a synteny study allowed the identification of putative tomato/pepper/eggplant orthologous QTLs/genes (Rinaldi et al., 2016). In 2016, the H2020 G2P-SOL project (www.g2p-sol.eu) started, aimed at bringing together most of the major European (CGN, INRA, IPK, UPV) and Asian (AVRDC) gene banks, Universities and Research Centres with the purpose to make an inventory of solanaceous species including most of the eggplant genetic material available. In total, 2,912 *S. melongena*, 305 *S. aethiopicum* and 122 *S. macrocarpon* accessions as well as a set of 266 accessions belonging to 29 wild species have been collected and inventoried (Giuliano et al., 2018). DNA was extracted from all accessions and The Single Primer Enrichment Technology (SPET) genotyping, recently developed by Nugen® was applied for their targeted genotyping (Barchi et al., 2019b). Starting from thousands polymorphic sites found on both coding regions and the introns/UTRs genome space, a panel of about 25K high confident SNPs evenly distributed on the genome were detected and a set of 5k best performing SPET probes was used for diversity analyses. The genotyping shed light on the worldwide genetic diversity and population structure of these crops and the extent of duplication between different gene banks. The Fast STRUCTURE analysis identified 9 main clusters (K), with *Solanum melongena* accessions grouping into 6 sub-clusters. Three further clusters were found, including accessions belonging to *S. macrocarpon* and *S. aethiopicum* as well as species belonging to the subgenus *Solanum*, in good agreement with clustering obtained with ML phylogenetic tree. A whole of 1114 accessions were finally classified as admixed. (Barchi et al., 2019) The gathered information was used for the development of a core collection of 450 accessions for GWA studies.

Genetic maps

Studies on eggplant genome organization have received an increasing amount of interest in the last decades, turning it from a "genomic orphan species" to a crop with a high-quality genomic sequence available (Barchi et al., 2019). In the past, as in several other crops the low level of polymorphism within the cultivated eggplant germplasm required huge efforts in detecting markers exploitable for linkage mapping purposes (Stàgel et al., 2008; Barchi et al., 2019). In eggplant, several inter-specific genetic maps built up by applying pre-NGS (next generation sequencing) techniques (RFLP, AFLP, RAPD, SSR, etc.) were developed, both based on inter and intra-specific populations, with the former exploiting a higher genetic polymorphism, but being of minor relevance for marker-assisted breeding. The first interspecific eggplant genetic map was constituted in 2002 by Doganlar et al. using an F2 population of 58 individuals obtained by crossing a commercial line of *S. melongena* (MM738) and its wild relative *S. linnaeanum*. Globally, 233 RFLP markers were tested on the population producing a molecular map of 12 LGs with a length of 1.480 cM. The RFLP exploited had a known map position in tomato which allowed the alignment of the chromosomes of the two species. The same map was improved by adding 110 COSII and 5 RFLP (Wu et al., 2009) previously mapped on tomato. Exploiting these bridge markers, 552 COSII markers with a known map position in tomato were also putatively assigned to the eggplant LGs (Doganlar 2009). Another interspecific map between cultivated eggplant and *S. incanum* was instrumental for mapping functional genes for fruit quality as well as for integrating tomato and other eggplant maps using common markers (Gramazio et al., 2014) The first intra-specific genetic map has been published by Nunome et al. (2001) who exploited 168 individuals from an F2 cross between two *S. melongena* L lines (cv. EPL- 1 a Japanese commercial line and WCGR112-8 a line which showed high resistance to the *Pseudomonas solanacearum* pathogen). A total of 181 dominant markers (of which 93 AFLP and 88 RAPD) were mapped on 21 LGs with a total length of 716.9 cM. Using 120 individuals of the same progeny, Nunome et al. (2003) produced a second map with 162 markers (7 SSR, 97 RAPD and 58 AFLP) which consisted of 17 LGs with a length of 716.9 cM. A third map (Nunome et al., 2009) resulted from the genotyping of 94 individual with 245 SSR markers resulting in 14 LG with a total length of 959.1 cM. The discrepancy between the number of LGs found out and the number of haploid chromosomes of *S. melongen*a indicates the low markers coverage of the genome obtained in these studies.

A F2 mapping population have been developed by Barchi et al. (2010) which consisted of 141 individuals produced from an intra-specific cross between two lines: 305E40, which carries a chromosome portion from the allied species *S. aethiopicum* conferring resistance to the pathogen

Fusarium oxysporum f.sp. *melongenae* and 67/3, a line susceptible to this fungus. Using 238 markers (including 212 AFLPs and 3 CAPs designed on the *Rfo-sa1* locus which confers resistance to *Fusarium*, 1 RFLP and 22 SSR) a molecular map was obtained consisting of 12 major and 2 minor LGs (Barchi et al., 2011). The advent of NGS-based marker technologies, by increasing the speed, throughput, and cost effectiveness of genotyping and providing genome-wide marker coverage, has allowed the development of the so called 'second generation' maps. The first next-generation map was developed by Barchi et al. (2012), which sequenced two RAD tag libraries of the two parental lines of the F2 mapping population 305E40 x 67/3. The alignment of this data yielded~10,000 SNPs markers, almost nearly 1,000 indels and 1800 SSR. A set of 347 *de novo* SNPs, together with 84 anchoring markers were applied to the F2 mapping population to construct a linkage map. In all, 415 of the 431 markers were assembled into 12 major and 1 minor linkage groups, spanning 1,390 cM, and the inclusion of established markers allowed each linkage group to be assigned to one of the 12 eggplant chromosomes (Fig 4). A blastN search against the tomato genome revealed that 223 of the SNP loci satisfied the cut off criterion applied, and thus, together with the RFLP and COSII markers (for a total of 261 loci), were suitable for assessing synteny and collinearity between the eggplant and tomato genomes.

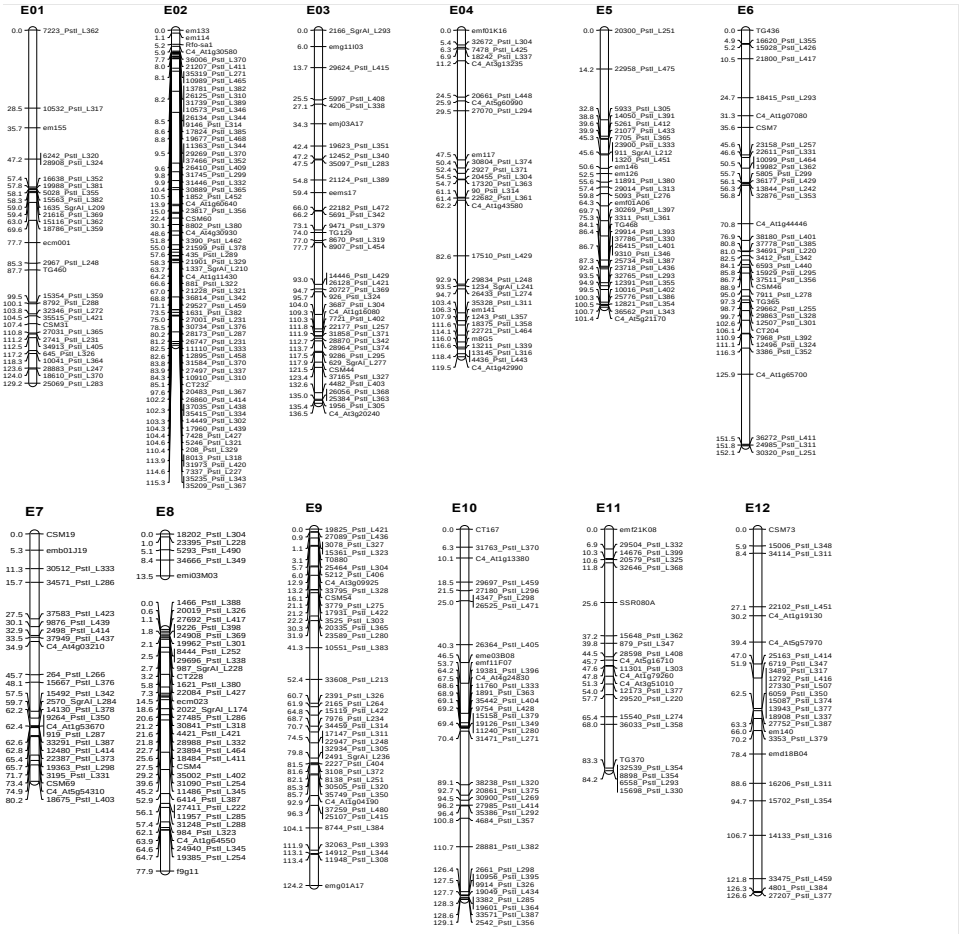

Figure 4 Eggplant molecular map proposed by Barchi et al. (2012)

Second generation intra-specific genetic maps were also generated for anchoring the first draft genome sequence of eggplant (Hirakawa et al., 2014) and for mapping resistance QTLs to *Ralstonia* strains by SNPs developed through Illumina sequencing of the parents of a RIL mapping population as well as AFLP, SSR and SRAP markers (Salgon et al., 2018). Fukuoka et al. (2012) used two intra-specific F2 mapping populations (LWF2 population 90 individuals and the ALF2 population 93 individuals derived from crosses between *S. melongena* LS1934 and *S. melongena* WCGR112-8 and between *S. melongena* AE-P03 and LS1934, respectively) to build a linkage map which consisted of 12 linkage groups and encompassed 1,285.5 cM in total. 952 DNA markers were mapped, including 313 genomic SSR markers developed by random sequencing of simple sequence repeat (SSR)-enriched genomic libraries, and 623 single-nucleotide polymorphisms (SNP) and insertion/deletion polymorphisms (InDels) found in eggplant-expressed sequence tags (ESTs). Among the used markers 326 could also be mapped onto the tomato genome.

Despite the recent efforts, the linkage maps used for identifying the genetic basis of traits of breeding interest were still not saturated, hampering the fine mapping of QTL regions and the identification of candidate genes associated with the phenotypic traits. The first high-resolution SNP-based linkage map was developed on a F2 population from the inter-specific cross (*S. melongena* x *S. linneanum*)

and employed to highlight QTLs affecting stem height and fruit and leaf morphology (Wei et al., 2020). More recently, a new intraspecific high-resolution linkage map based on the RIL mapping population from the cross 305E40x67/3 was developed (Toppino et al., 2020), including 7,249 SNPs assigned to 12 chromosomes and spanning 2,169.23 cM, with an average distance of 0.4cM between adjacent markers.

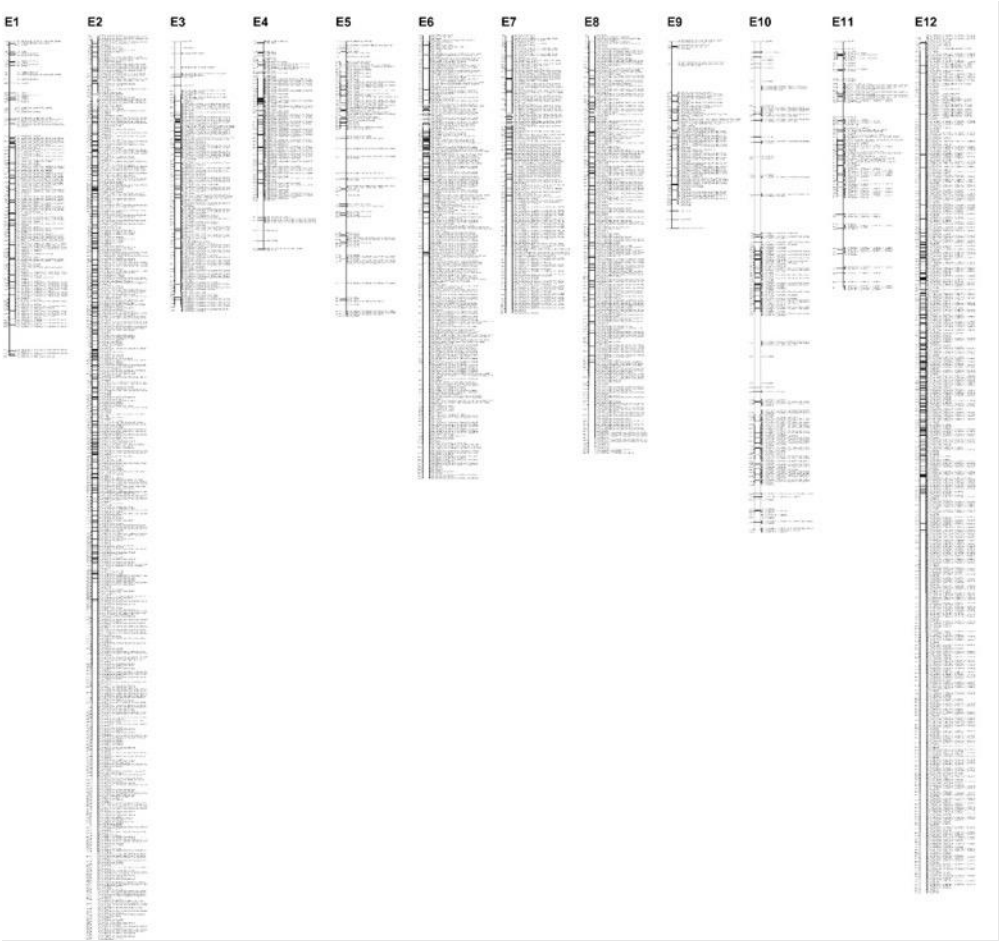

Figure 5: Eggplant linkage map. Marker names are shown to the right of each chromosome, with map distances (in cM) shown on the left.

Eggplant Quantitative Trait Loci (QTLs)

In general, linkage maps developed were pivotal for QTL mapping of morphological, agronomical and biochemical traits. Although the huge phenotypic variability available in *S. melongena* accessions, the reduced polymorphism detected in intraspecific mapping populations with the markers used (i.e. RFLP, RAPD, AFLP) in the "first generation" maps hampered the construction of dense eggplant genetic maps. As a result, the identification of QTLs in this species was limited, especially when compared to the other Solanaceae crops as tomato, potato and pepper.

The first QTLs mapped in eggplant by Nunome et al. (2001) were related to fruit shape and color of fruit, stem and calyx. Doganlar et al. (2002b) phenotyped 22 traits, including those related to fruit weight, fruit shape, and color, the F2 interspecific population from the cross between *S. melongena* and *S. linneanum*, and a total of 47 unique QTLs were identified. Later, the same population was used to map chromosomal regions associated to 19 morphological and biological traits, including leaf, flower, fruit characteristics, day to flowering and fruit set and resistance to abiotic and biotic stresses (Frary et al. 2003). The same phenotypic traits data were re-analysed (Frary et al. 2014) using a more performant method and a more dense map and 71 QTLs were identified, of which 22 were novel ones (Doğanlar et al. 2014).

QTLs associated to parthenocarpy, a very important fruit qualitative trait, were identified by Miyatake et al. (2012), by exploiting the improved maps of Fukuoka et al. (2012).

The first intraspecific map developed by Barchi et al. (2010) allowed the positioning of the *Rfo-sa1* locus for the resistance trait to *Fusarium oxysporum* in the upper part of chromosome 2. The second-generation map constructed using the same F_2 population (Barchi et al. 2011) was validated by mapping QTLs linked to traits related to anthocyanins pigmentation and distribution on leaf, stem, flower and fruit peduncle. The same mapping population was phenotyped in replicated blocks in two locations and QTL mapping led to the identification of chromosomal regions associated with more than 70 traits including the agronomical relevant features of early and total yield, fruit weight and shape, prickliness in calyx and leaf (Portis et al. 2014). A biochemical characterization for both fruit qualitative traits, including dry matter, brix, sugars, and organic acids, as well as for health-related compounds, including chlorogenic acid, the two peel anthocyanins [i.e., delphinidin-3-rutinoside (D3R) and delphinidin-3-(p-coumaroylrutinoside)-5-glucoside (nasunin, *Nas*)] and the two mains steroidal glycoalkaloids, (solasonine, and solamargine) was also carried out on the same population (Toppino et al, 2016).

For most of the traits, at least one major QTL was identified; a total of nearly 60 major (PVE>10%) and many other minor QTL were spotted; major QTLs were stable in the two locations, while

location-specific QTLs were solely represented by minor QTL. Finally, putative syntenic orthologous genes with other *solanaceous* species were identified. Furthermore, through a GWAs approach, the previously identified loci were validated and a number of new marker/trait associations detected (Cericola et al., 2014; Portis et al., 2015)

Resistances to pest and diseases have been the targets of most QTLs studied in the Solanaceae (Gebhardt 2016). An intraspecific RIL population was developed for mapping a major resistant locus to *Ralstonia solanacearum* (Lebeau et al. 2013) and later, for mapping the resistance against eight strains by using a high density GBS-based map with 1,035 markers and anchored on eggplant, tomato, and potato genomes (Salgon et al. 2017).

Recently a new intraspecific mapping population from the cross MM 738 (susceptible) x AG91-25 (resistant to a broad range of *R. solanacearum* strains) was genotyped by GBS and generated a high dense map of 1,170 (Salgon et al. 2018) and used to map new QTLs associated to resistance.

Fusarium oxysporum f. sp. *melongenae* and *Verticillium* spp. are the most dangerous soil-borne fungal wilt disease causing heavy yield losses in eggplant cultivation. The map position of the locus *Rfo-sa1* associated with the resistance trait to *Fom* was firstly established on the last portion of chromosome E02 using the second-generation map of the segregant F_2 population from the cross 305E40x67/3 (Barchi et al. 2012 and 2018; Portis et al. 2014); the same mapping population was then employed for spotting two major QTLs associated with the resistance to *Fusarium* (one on chromosome E02, the other one, from the 67/3 parent, on E11) and also to spot for the first time both major and minor QTLs associated with the partial resistance to *Verticillium dahliae* (Barchi et al., 2018). Another work on *Fusarium* wilt resistance was performed using three *S. melongena* resistant sources lines (Miyatake et al. 2016). In this work, precise mapping was accomplished by using three genetic maps from F_2 and F_3 populations as well as backcross inbred lines populations and revealed a semi-dominantly inherited QTL locus (*FM1*) positioned on chromosome 2 at the same location of *Rfo-sa1*, suggesting they might be orthologous.

Recently, the RIL population developed from the intraspecific cross ‘305E40’, x ‘67/3’ whose genome sequence was recently released) employed for developing the first GBS based linkage map of eggplant, was used to elucidate the genetic bases of seven traits related to anthocyanin content in different organs recorded in three locations as well as seed vigour (Toppino et al., 2020). Overall, from 7 to 17 QTLs (at least one major QTL) were identified for each trait, confirming that the newly developed map supply valuable information for QTLs fine mapping, candidate gene identification, and development of molecular markers for the MAS selection of favourable alleles.

The bridge between the genetic map and assembled genome sequence are the QTLs studies which then enables the identification of candidate genes in the region surrounding the QTL. Moreover,

QTL's characterization is a vital tool in marker-assisted plant breeding programs, enabling plant breeders to develop new lines and varieties in response to demands such as increased nutraceutical and marketable value of fruits.

The Eggplant Genome

Like tomato potato and pepper, eggplant is a prevalently autogamous diploid species with 12 chromosomes (2n=2x=24). The eggplant chromosome number is the same in all varieties, but chiasma frequency during diplonema and diakinesis shows varied bivalents (Simmonds, 1993). The eggplant nuclear genome contains about 1,14 Gb of DNA and it is larger than tomato (740 Mb) and diploid potato (1,000 Mb) ones, bus smaller than the genome of pepper (2,200 – 2,700 Mb) (Arumuganathan and Earle, 1991). The first eggplant genome draft sequence of the typical Asian eggplant cultivar Nakate-Shinkuro was released in 2014 (Hirakawa et al., 2014), although scarcely annotated and only partially (12%) anchored to a genetic map. The non chromosome-anchored draft sequence of this eggplant genome dataset is composed by 33,873 scaffolds that cover 833.1 Mb, the 74% of the eggplant genome. More recently, a chromosome-anchored draft of eggplant genome was assembled by an Italian consortium (Barchi et al., 2019) using the line '67/3', a breeding line developed at CREA. The hybrid assembly, covering 1.22 Gb, was obtained by merging Illumina sequencing data and optical mapping. The female parent of the RIL mapping population (line '305E40') was also sequenced (coverage of 34X), and thanks to low coverage resequencing (1X) of the F6 RIL population a physical map of the assembled genome was obtained. In detail, 469 scaffolds were identified and anchored on chromosomes using the SoiLoCo pipeline (Scaglione et al 2016) and a RIL mapping population (Barchi et al. 2012). In addiction RNA-Seq from 19 tissues of "67/3" allowed to identify 34,916 high-quality protein-coding gene models providing a useful resource for functional genetics studies. Recently, based on Illumina sequencing data, a draft genome assembly of 1.02 Gb in size was developed for the cultivated species *S. aethiopicum* (Song et al., 2019), which showed like in eggplant, about 76% of repetitive sequences. Furthermore, in respect to *S. melongena*, an expansion of gene families involved in drought or salinity tolerance as well as disease resistance including defense response was identified. In 2020 a new high-quality genome for the eggplant inbred line HQ-1315 (*S. melongena*-HQ) using a combination of Illumina, Nanopore and 10X genomics sequencing technologies and Hi-C method for genome assembly was released (Wei et al., 2020). Genomes are becoming a compulsory tool for the identification of candidate genes for important traits in eggplant. Recently, the resequencing of seven eggplant accessions and one accession of the wild relative *S. incanum,* which are the parents of a MAGIC population, has been performed by Gramazio et al. (2019). The set of identified SNPs polymorphisms has been annotated and currently is being

used for further analyses in order to efficiently genotype the MAGIC population with the goal to dissect key agronomic and morphological traits. More recently, 60 genotypes of the cultivated scarlet eggplant (*S. aethiopicum*) belonging to the varietal groups, “Gilo” and “Shum”, as well as 5 accessions of its ancestor *S. anguivi*, were sequenced at coverage of 30-60X, with the goal to investigate the evolution, population demography and domestication history of the species (Song et al 2019).

Molecular breeding strategy

Selection for important quantitative traits is traditionally based in animals and plants on phenotypic records of the individual and its relatives. The method of Henderson (1984) follows the idea that the phenotype is determined by the sum of the genotype effect and the casual deviations due to the environment. If a wide range of observations is made it is possible to assume that the deviations caused by environment are normally distribute with a sum equal to zero. In this case the phenotypic value is a valid estimation of the genotypic value of an individual which is the hereditable component of its observed variability. Reliable evaluation of this value requires extensive effort in phenotyping trials which often result in prohibitively expensive costs. In the 1980s the molecular biology started to use the natural occurring DNA variations as markers to tag chromosome portions. The information at the DNA can be captured as a molecular score that can be used for selection of some chromosome portions associated with a trait of interest. This approach led to faster genetic gain than the one achieved based on phenotypic data only. The inclusion of markers information into breeding values was predicted to yield 8-38% extra genetic gain in breeding programs (Meuwissen et al., 2001).
Moreover, the possibility to follow correlated segregation of markers in artificial progenies made it possible the so called "linkage analysis", which allowed to obtain "linkage maps" or "genetic maps", i.e., ordered groups of markers which are a representation of chromosomes. Extensive efforts in improving experimental designs and statistical tools made it possible to combine morphological and genotypic data to detect the position of Quantitative Traits Loci (QTLs) of agronomic interest, and identifying markers closely linked to loci bearing the desirable features.
An alternative to biparental linkage mapping for determining the genetic basis of trait variation is represented by the genome-wide association study (GWAS) mapping approach. Both approaches rely on recombination to re-arrange the genome, and seek to establish correlations between phenotype and genotype, based on the non-random association of alleles at two or more loci, termed linkage disequilibrium (LD). The major advantages of GWAS lie in being able to sample a much wider range of the phenotypic and genotypic variation present in many different lineages, in exploiting multiple

rounds of historical recombination, and in including multiple accessions of direct relevance to crop improvement (Yu et al. 2006).

In a pioneering attempt to apply a GWAS approach in eggplant, Ge et al. (2013) were able to identify a number of phenotype/genotype associations related to eight fruit-related traits. Subsequently, the analyses of a large association panel and SNP data set were performed with the aim to identify and position marker/trait associations related to fruit, plant and leaf morphological traits relevant for eggplant breeding (Cericola et al. 2014; Portis et al. 2015). Employing the same SNP loci reported by Barchi et al. (2011), 339 of these being previously genetically mapped (Barchi et al. 2012). The major outcome of these approaches is to delivery essential tools for Marker-Assisted Selection (MAS), allowing a faster and robust introgression of desirable QTLs alleles from one variety to another, or to incorporate interesting chromosomal segments from wild germplasm into breeding material.

Marker assisted selection (MAS)

The plant breeding has the goal to improve the performance of the crops. One of the aim is to incorporate a desired gene/genetic locus for an agronomic trait (e.g. a resistance trait to pathogen, or the peel color of fruits) from a donor into an elite cultivar or breeding line (the recurrent parent) in a procedure called “introgression” or “recurrent selection” through backcrossing and selfing of the selected plants. The desired outcome is a line containing only the major gene from the donor parent, which generally is a wild species with poor agronomical value (especially when introgressing resistance traits), introgressed into the genetic background of the recurrent parent genotype (Hospital, 2005). Otherwise, a superior material can be developed through the combination of genes which are carried by several improved lines.

Marker-assisted selection represents a procedure aimed to accelerate the breeding efforts, using markers closely linked to the QTLs/genes to scan the progeny of each breeding cycle.

This procedure allows to make the plants selection in the early stages of their life cycle without performing phenotyping characterizations which are highly expensive and time consuming.

The efficiency of marker-assisted selection depends on a number of factors: one of them is the population size of each generation considered, as the larger is the population at each step of selection, the higher is the probability to find the desired allelic combination. The type of the marker used is also a key parameter: desirable characteristics of a good molecular marker include co-dominant inheritance, reproducibility, low cost of marker genotyping, and capacity to be run on a high-throughput scale. For all these reasons, SNPs are currently in wide-spread use in molecular marker applications (Khan et al., 2007).

The distance of markers from the target locus is another fundamental parameter to be considered: the more the marker is physically close to the gene responsible of the trait of interest, the fewer recombinations will occur between the two loci at each round of crossing thus reducing the number of false positives selected. The best option to avoid any recombination event, when possible, would be to develop the marker within the gene sequence (Song et al., 2003; Colton et al., 2006). As alternative, researchers have promoted selection based on markers that flank the locus or gene at each side (flanking markers) (Soller and Plotkin-Hazan, 1977; Tanksley and Hewitt, 1988; Hospital and Charcosset, 1997). By selecting on the basis of the marker genotype of flanking markers, breeders can identify recombination events between one of the markers and the gene. If there was a cross-over between a marker and gene, the two markers, which are on either side of the gene, would have different genotypes. The chance that double crossovers occurred is small and related to the distance between flanking markers (distance between marker and gene and distance between gene and the second marker).

MAS examples in *Solanaceae*

While many marker/trait association studies have been conducted in *Solanceous* crops, only a few of these markers are being used for MAS due to cost of the genotyping system, population specificity of the associations, linkage distance between the marker and the gene, and the amount of phenotypic variation the markers account for (Felcher, 2012).

However, since the knowledge of the genomes of these species grows allowing genes identification and since the cost of the marker genotyping is becoming less prohibitive, MAS will almost certainly become an increasingly important tool used by breeders. Although several works have been focused on the identification of markers linked to agronomic important traits, at present, only a few markers have been employed for MAS in eggplant (Mutlu et al., 2008; Toppino et al., 2008; Cao et al., 2009; Liao et al., 2009). More extensive examples of how MAS can be used to improve crops are reported for tomato. Robbins et al. (2009) used a population derived from several BC and selfing cycles to identify a marker closely linked to the tomato bacterial spot resistance which subsequently was incorporated into the plant selection. (Yang and Francis, 2005) published the result of a successful pyramiding of two resistance genes respectively for the bacterial spot and the bacterial speck plant pathogens into new homozygous lines. These lines were selected by means of a two markers screening carried out on a F2 population derived from the cross of two cultivar each baring one of the two resistances.

Molecular markers in eggplant

In eggplant, the first reports regarding genetic molecular markers were published on the application of RAPD (Karihaloo et al. 1995), AFLP (Mace et al. 1999. Furini et al. 2004) and SSR (Nunome et al 2003a, b) markers and studies on genetic map construction based on AFLP and RAPD markers (Nunome et al.2001) or tomato cDNA, genomic DNA and EST markers (Doganlar et al. 2002). A first subset of 50 SSR markers was developed by Stagel et al. (2008) from 3,300 eggplant genic DNA sequences available in the Solanaceae Genomics Network (SGN) and EMBL databases, which were then applied to a panel of 44 Solanum genotypes, in order to assess their informativeness. Furthermore, Nunome et al. (2009), through the construction of SSR-enriched genomic and cDNA libraries, identified more than 1,000 SSR markers of which 221 segregated in an intra-specific mapping population and were applied for the construction of the derived genetic linkage map. In 2011, Barchi et al. provided the first SNPs panel in eggplant based on the RAD tag sequencing. A RAD library was generated from the genomic DNA of a pair of eggplant mapping parents (more details in "Genetic maps in eggplant" chapter) at the Floragenex Inc. according to the protocol described by Baird et al. (2008). The sequencing of the library produced nearly 17.5 Mb of sequences arrangeable into nearly 78,000 contigs. The resulting non-redundant genomic sequence dataset consisted of about 45,000 sequences. The shared sequences allowed the discovery of nearly 10,000 SNPs and nearly 1,000 indels. A subset of 384 SNPs was used to successfully fingerprint with the Goldengate assay a panel of eggplant germplasm. The RAD sequences also included nearly 2,000 putative SSRs, and primer pairs were designed to amplify 1,155 loci. Thanks to the recent availability of the 67/3 high quality whole genome sequence, Portis et al. (2016) identified nearly 133,000 perfect SSRs, with a density of 125.5 SSRs/Mbp, and also about 178,400 imperfect SSRs. Di- and tri-nucleotide SSRs accounted, respectively, for 43 and 37% of the total. The SSRs were classified according to their number of repeats and overall length and were then assigned to their linkage group. Putative functions were assigned via ontology to genes containing at least one SSR. Using this data, an "Eggplant Microsatellite DataBase" (EgMiDB) was developed, which permits identification of SSR markers in terms of their location on the genome, type of repeat (perfect vs. imperfect), motif type, sequence, repeat number and genomic/gene context and also suggests forward and reverse primers. An "in silico" PCR analysis was performed to validate these SSR markers, using as templates two CDS sets and three assembled transcriptomes obtained from diverse eggplant accessions.

A widely used method for high-throughput SNP discovery and genotyping is Genotyping by sequencing (GBS) based on different reduced-representation sequencing (RRS) approaches, the majority of which are based on the use of restriction enzymes. Recently, 163 Recombinant Inbred

Lines (RILs) from the intraspecific cross 305E40x67/3 were genotyped by a GBS approach, able to detect 10,361 polymorphic sites (Toppino et al., 2020). Overall, 267Gb of sequencing data were generated and ~773M Illumina paired-ends (PE) reads were mapped against the reference sequence. One major limitation of GBS is the random distribution of restriction enzyme sites on the genome, and thus the inability to target markers localized within genes or having a functional significance.

Recently Nugen® developed the Single Primer Enrichment Technology (SPET, US Patent 9,650,628), which is a customizable solution for targeted sequencing at an affordable price which requires a priori genomic or transcriptomic information and identification of SNPs for probe design. In order to assess the potential of SPET for high-throughput genotyping in plants, a panel comprising 5k target SVs, designed both on coding regions and introns/UTRs, was developed for tomato and eggplant. Genotyping of a panel composed of 422 eggplant accessions, comprising both domesticated material and wild relatives, generated a total of 30,731 high confidence SVs, which comprised both target and novel SVs. The vast majority of the markers was transferrable to closely related wild species. In the last decade many efforts have been made to accumulate molecular genetic information such as expressed sequence tag (EST) resulted in the identification of 98,086 sequences of which 16,245 unique sequences. The dataset includes eggplant transcriptome from various accessions and tissue at different developmental stages (Fukuoka et al. 2010).

Anthocyanins

Characteristics and distribution

Anthocyanins are a group of polyphenolic pigments ubiquitously found in the plant kingdom, belonging to the group of flavonoids. Flavonoids are a subtype of secondary metabolites which are accumulated in the vacuole of plant cells and are the main responsible for the tissue pigmentation in higher plants. They are readily recognizable as floral pigments in most of the angiosperms, with a wide spectrum of colors ranging from yellow to blue, but they are also present in other tissues and organs of the plant, especially in fruits, roots and leaves. The chemical structure is the flavinic nucleus formed by two aromatic benzene rings (A and B rings) linked via a heterocyclic pyranic ring with three carbon atoms (C ring). Flavonoids occur as aglycones, glycosides, and methylated derivatives. In relation to the modifications of C ring (mainly oxidation reactions), flavonoids have been divided into different structural classes namely flavones, isoflavones, chalcones, aurones, flavanones, flavonols, catechins, leucoanthocyanidins and anthocyanins. Chemically, anthocyanidins are flavylium cations and are generally present as chloride salts, which occur like glycosides of anthocyanidins (aglycons) (Konczak & Zhang, 2004; Tanaka et al., 2008; Stommel et al., 2009). There is a huge variety of anthocyanins in nature differing from each other by the number of hydroxylated groups, the nature and the number of bonded sugars to their structure, the aliphatic or aromatic carboxylates bonded to the sugar in the molecule and the position of these bonds (Kong et al., 2003). Up to now, more than 600 different anthocyanins were discovered (Andersen & Jordheim, 2006) and more than 20 anthocyanidins, but only six of them are ubiquitous in plants: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (Zhao et al., 2014; Clifford, 2000). The distribution of the six most common anthocyanidins in fruits and vegetables is: cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%) (Kong et al., 2003). Pelargonidin, cyanidin, and delphinidin are considered primary anthocyanidins and differ from each other by the number of hydroxyl groups on the B ring. They are responsible for orange/red, red/magenta and violet/blue pigmentation, respectively (Tanaka and Ohmiya, 2008). The primary anthocyanidins are the most common in nature, being found in 80% of pigmented leaves, 69% in fruits and 50% in flowers (Dey & Harborne, 1993). The secondary anthocyanins occur by single or double methylation of delphinidin which result in petunidin and malvidin formation, respectively. Moreover, secondary anthocyanins can occur from a single O-methylation of cyanidin, which allows the formation of peonidin.

Biological function of anthocyanins

Anthocyanins are secondary metabolites belonging to the flavonoid class characterized by the presence of chromophores. The color exhibited by these molecules was first explained by Pauling in 1939, who proposed that the resonant structure of the flavylium ion caused the intensity of their color (Wrolstad et al., 2005). Anthocyanins are ubiquitously distributed among the higher plants and are responsible for colors varying from orange, red, and purple to blue in flowers, seeds, fruits and vegetative tissues (Tanaka and Ohmiya, 2008). Anthocyanins play an important role in facilitating plant reproduction as they attract pollinators and seed dispersers by imparting bright colors (Harborne and Williams, 2000; Hoballah et al., 2007). Besides, anthocyanins show many chemical effects on plants, as they protect plants from several biotic and abiotic stresses (Chalker-Scott, 1999). Their presence can reduce the infestation of insects and pathogens as well as enhance tolerance to heat stress (Malone et al., 2009; Zhang et al., 2013; Meng et al., 2015). Anthocyanins may also play an important role to improve the postharvest characteristics of vegetables. In tomato fruits, for example, anthocyanins prevent lipid peroxidation, reduce the over-ripening and enhance a longer shelf-life (Jiao et al., 2012; Bassolino et al., 2013). Therefore, it is also known that anthocyanins perform photoprotective roles acting like a light-absorbing screen (Hughes et al., 2005; Albert et al., 2009) and protecting chloroplasts from the photo-oxidative and photo-inhibitory damage by scavenging free radicals and reactive oxygen species (ROS) (Gould, 2004). Anthocyanins have a higher antioxidant activity than other flavonoids due to their positively charged oxygen atom (Kong et al., 2003). In general, the antioxidant activity of anthocyanins depends on the degree of hydroxylation at the B-ring as well as the type and extent of acylations and glycosylations. Hydroxylation at the B-ring enhances antioxidant capacity, on the other hands, the glycosylation decreases the antioxidant activity with the number of glycosyl groups attached to the A and C ring (Sadilova et al., 2006). In line to what above-mentioned, plants need sophisticated regulatory mechanisms to ensure that the degree of anthocyanin pigmentation is appropriate to myriad developmental and environmental signals (Albert at al., 2014). To fulfil these roles, stringent regulatory mechanisms must exist that respond to environmental and developmental cues.

Anthocyanin biosynthesis

Anthocyanins are synthetized through a branching of the phenylpropanoid pathway, which is one of the most studied networks in higher plants and it is highly conserved among species. In dicotyledonous species, the structural genes coding for enzymes involved in the biosynthetic pathway of anthocyanins are commonly divided into two clusters: i) the early biosynthetic genes (EBGs)

coding for the enzymes Chalcone Synthase (CHS), Chalcone Isomerase (CHI) and Flavanone 3-Hydroxylase (F3H) which control the common "early" steps leading to the production of many flavonoids (including the yellow pigment naringenin) necessary for the anthocyanins biosynthesis; ii) the late biosynthetic genes (LBGs) coding for the enzymes Flavonoid 3'-Hydroxylase (F3′H), Flavonoid-3'-5'-Hydroxylase (F3′5′H), Dihydroflavonol 4-Reductase (DFR), Anthocyanin Synthase (ANS) and, finally, the "decorating enzymes" playing role in the specific production of different anthocyanins (Liu et al., 2018). The anthocyanin biosynthetic pathway starts from the general flavonoid pathway, with the conversion of phenylalanine in 4-cumaroil-CoA performed by the enzymes Phenylalanine Ammonia Lyase (PAL), Cinnamate 4- Hydroxylase (C4H) and 4-CoumarateCoA Ligase (4CL). The 4-cumaroil-CoA is the starting substrate to produce many compounds including volatile compounds, lignin and flavonoids. The anthocyanin branch starts with the Chalcone Synthase (CHS) which catalyses the conversion of 3xMalonyl CoA and β-Coumaroyl-CoA to the yellow pigmented naringenin chalcone; then, Chalcone Isomerase (CHI) converts naringenin chalcone to naringenin and Flavanone 3-Hydroxylase (F3H) converts naringenin into Dihydrokaempferol (DHK) that can be further hydroxylated by F3′H and F3′5′H into two other dihydroflavonols, namely Dihydroquercetin (DHQ) or Dihydromyricetin (DHM), respectively. Next, the Dihydroflavonol 4-Reductase (DFR) converts the dihydroflavonols (DHK, DHQ and DHM) to their respective leucoanthocyanidins; then Anthocyanin Synthase (ANS) catalyses the conversion of leucoanthocyanidins to anthocyanidins. Finally, sugar molecules and acyl groups are attached to anthocyanidins by various members of the glycosyltransferase family, which are commonly called "decorating enzymes". Most of the naturally occurring flavonoids are glycosylated in 3' position. Indeed, the most studied flavonoid glucosyltransferase are the Flavonoid 3-O-Glucosyltransferases (3GT). The 3GT enzymes have been characterized biochemically and molecularly in maize (Zea mays; Futtek et al., 1988), snapdragon (Antirrhinum majos; Martin et al., 1991), grape (Vitis vinifera; Ford et al., 1998) and gentian (Gentiana triflora; Tanaka et al., 1996). Moreover, others 3GT frequently occurs: anthocyanidin 3-O-galactosyltransferase, characteristic of beans and the anthocyanidin 3-O-glucoside rhamnosyltransferase common in many Solanaceae species (Holton and Cornish, 1995). Afterwards, generally an anthocyanin acyltransferase (AAT) and a flavonoid 5-glucosyltransferase (5GT) occur in *Solanaceae* (Provenzano et al., 2014). In petunia flowers, mutation of *aat* gene results in the accumulation of anthocyanidin 3-rutinosides which most likely indicates that the functionality of 5GT depends on AAT activity (Jonsson et al., 1984). Moreover, in *Solanaceae*, the "decoration enzymes" glycosyltransferases are highly conserved, demonstrating a relatively early evolution, before the divergence of the *Solanaceae* family, with respect to AAT which appears to have arisen after divergence of the Solanaceae (Tohge et al. 2015).

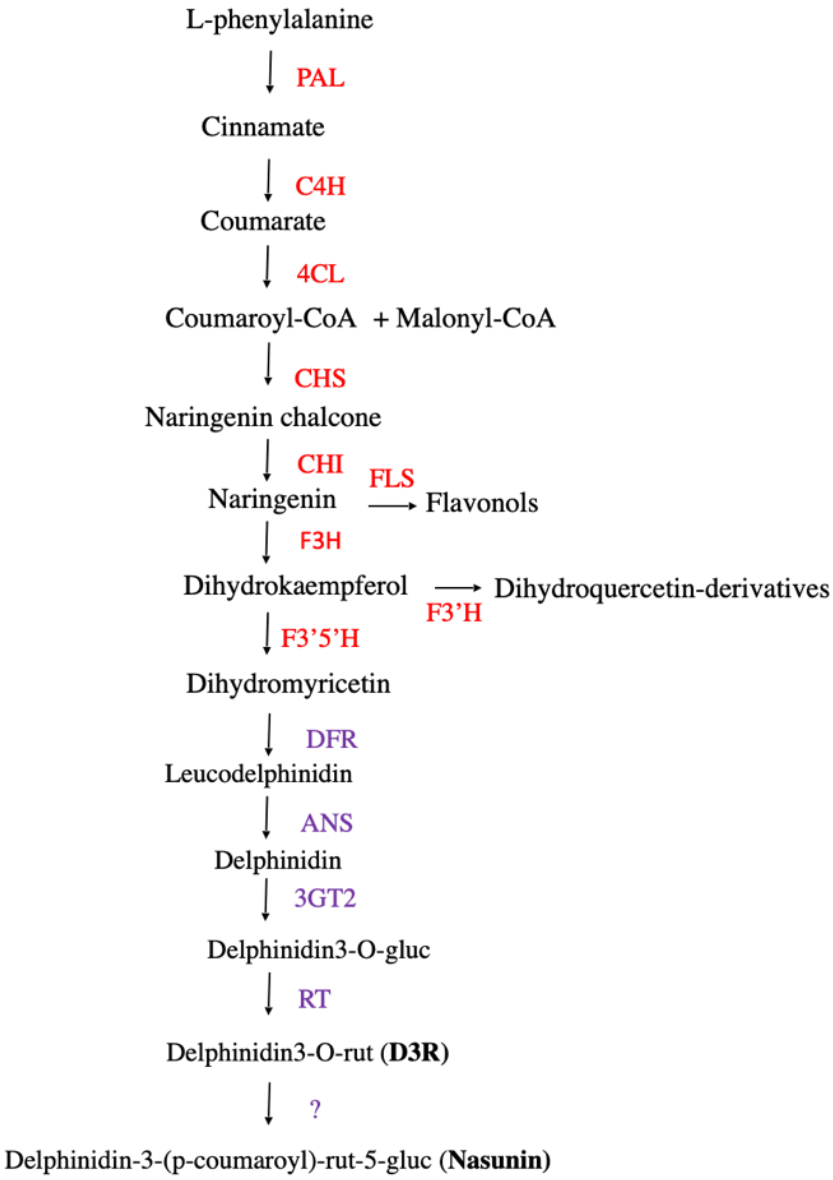

Figure 6: Anthocyanins pathway in eggplant. In red and violet EBGs and LBGs respectively.

Anthocyanins regulatory genes

The regulatory networks of anthocyanin biosynthesis are an important model for understanding how metabolic pathways are controlled in plants and how the involved genes expression occur. In particular, the expression of the anthocyanin pathway related genes is regulated by many transcription factors (TFs) which have been identified and well described in many species, including petunia (*Petunia hybrida*), tomato (*Solanum lycopersicum*), snapdragon (*Antirrhinum majus*), *Arabidopsis thaliana* and maize (*Zea mays*) (Quattrocchio et al., 1998, 1999; Walker et al., 1999; Spelt et al., 2000, 2002; Mathews et al 2003; Carey et al., 2004; Schwinn et al., 2006; Albert et al., 2011).

Different R2R3-MYB TFs have been described to be implicated in regulation of anthocyanin biosynthesis by activating the transcription of anthocyanin structural genes in many species (Albert et al. 2011; Bovy 2002; Takos et al. 2006). In dicotyledonous species, the EBGs result regulated at the transcriptional level by a set of redundant MYB TFs, while the expression of LBGs is under the control of a transcriptional activation complex known under the name of "MBW complex" (Baudry et al., 2004; Koes et al., 2005; Ramsay and Glover, 2005) which is highly conserved among dicot and is composed by at least one member of each of the three gene families of R2R3-MYB, basic-helix-loop-helix (bHLH), and WD-repeat (WDR) proteins with the function of scaffold for both R2R3-MYB and bHLH. (Albert et al.2014). Recently, a group of MYBs which have role in the decrease or repression of the anthocyanins biosynthesis have also been identified (Parket al., 2008; Cao, et al., 2017; Colanero et at., 2018; Gates et al., 2018; Zhou et al., 2019; Chenet al., 2019; Moglia et al., 2020). The MBW complex has been well characterized in many *Solanaceae* species. Despite the knowledge availability is growing, anthocyanin metabolism is still mostly unknown for species like eggplant. However, the characteristics of MBW complex, found in the literature for this family are summarized and grouped into four groups: R2R3-MYB activators, bHLHs, WDRs (WD40) and the MYBs repressors.

R2R3-MYB Activators

The functions of MYBs genes have been identified in many eukaryotes including fungi, plants and vertebrates (Ohi et al. 1994; Martin and Paz-Ares 1997). The MYBs have roles in a wide range of cellular processes such as the regulation of secondary metabolism, the signal transduction, the cell division and the plants response to biotic and abiotic stress (e.g. pathogens, UV-B light, cold and drought) (Larkin et al. 1994; Borevitz et al. 2000; Cominelli et al. 2005). The MYBs are classified based on the strong conservation of the DNA-binding domain (Martin and Paz-Ares 1997). Different MYBs proteins are recognized to activate the transcription of many structural genes of different branches of the phenylpropanoid biosynthetic pathway. The promoters of these structural genes contain potential MYB protein recognition motifs (Martin and Paz-Ares 1997). Numerous TF's related to the MYBs family have been recognized as involved in activation or repression of the MBW complex. The patterning and spatial localization of anthocyanins are primarily determined by MYB activators mainly belonging to the R2R3-MYB family.

The R2R3-MYB possess the binding site for the nucleotide sequence of some LBGs promoter regions, basic helix-loop-helix (bHLH) domains and WD-repeat proteins domains (Albert et al., 2011; Lowry et al., 2012). Various regulation levels of anthocyanins production have been investigated, including the post translational modifications of MBW related genes (Xie et al., 2012;

Ye et al., 2012; Maier et al., 2013; Patra et al., 2013) and chromatin remodelling (Hernandez et al., 2007). Nesi et al. (2001) report the molecular and functional analyses of the *TT2* gene, encoding for a R2R3-MYB, from Arabidopsis, which resulted at least partially involved in the seeds pro-anthocyanidin accumulation. Therefore, two clades of genes encoding R2R3-MYB have been identified in *Solanaceae*, commonly named *ANT1* or *AN2* and *CaMYB* in pepper. In petunia, the *ANTHOCYANIN 2* (*PhAN2*) regulates the expression of LBGs in flower. The *AN2* expression seems restricted to the corolla limb but paralogs of *AN2* have been identified and apparently control pigmentation in different organs (Spelt et al., 2000); in tomato, the overexpression of two R2R3MYBs encoding genes *SlANT1* and *SlAN2*, allow the anthocyanin accumulation in different tissues, proving their involvement in the regulation of EBGs, LBGs and MBW related genes (Kiferle et al., 2015). Anthocyanin fruit (*Aft*) and atroviolacea (*atv*) loci were characterized in wild anthocyanic tomato. The *SlANT1* gene showed the best genetic linkage with the *Aft/Aft* mutation and revealed both nucleotide and amino acid polymorphisms between the *Aft/Aft* and cultivated genotypes (Meng et al., 2015; Kiferle et al., 2015). In pepper, *CaMYB* was shown by Borovsky et al. (2004) to encode for a MYB transcription factor homologous to petunia *AN2* uniquely expressed in anthocyanin fruits of the breeding lines 5226 and 06C59. Moreover, CaMYA can regulate the expression of both EBGs and LBGs (Liu et al., 2018) and might be involved in the defence response to pathogens (Zhang et al., 2015). In potato, the expression level of *StAN1* (named *StAN2* in some studies) seemed linked with the anthocyanin content (Jung et al., 2009; Payyavula et al., 2013), this was later confirmed through the overexpression of *StAN1* under the control of 35S promoter which allow the anthocyanins accumulation in potatoes peel and flesh (Jung et al., 2009).

MYB Repressors

Two distinct categories of MYB TFs have been identified that can repress the anthocyanin biosynthesis: R2R3-MYB and R3-MYBs repressors. The R2R3-MYB repressors contain a repression motif in their C terminus required for their activity, while R3-MYB repressors do not (Tamagnone et al., 1998; Jin et al., 2000). Besides, both types of MYB repressors are able to passively repress anthocyanin biosynthesis by competing with MYB activators to the binding of bHLH proteins in the MBW complex thereby reducing its activation capability. This passive-repressive mechanism is also known as “squelching” (Cahill et al., 1994). In petunia an R2R3-MYB repressor, the MYB27 was deeply characterized by Albert et al. (2014) which results involved in the MBW complex inhibition. The role of R3-MYBs in regulating flavonoid synthesis is less clear than for the R2R3-MYBs. Is known that the R3-MYB factors contain a single MYB domain and are typically small proteins which compete for bHLH partners with R2R3-MYB activator (Koes et al., 2005; Zhang et al., 2009). The

Arabidopsis *AtMYBL2* encode an R3-MYB (containing a single MYB repeat) but is an exception, because appears functionally similar to R2R3-MYB repressors (Kranz et al., 1998) as it contains a repression motif (TLLLFR) present in its C terminus which is required for its repressive activity (Dubos et al., 2008; Matsui et al., 2008). In petunia the homologous *MYBx* has been identified and involved in the feedback regulatory network to fine tune the anthocyanin accumulation (Kroon, 2004; Albert et al., 2011). In tomato the deep investigation of *Anthocyanin fruit* (*Aft*) and *Atroviolacea* (*atv*) loci recently allowed the identification of two MYBs with repression activity of anthocyanin synthesis. The two genes are the *SlAN2-like* and *SlMYB-ATV* an R2R3MYB and an R3MYB respectively. The *SlAN2-like* enhance the *SlMYB-ATV* which acts as repressor fine-tuning the accumulation of anthocyanins in tomato fruit (Colanero et al., 2018, 2020). Despite the increased interest and the result recently scored by the scientific community, the anthocyanins repressors topic still remains unknown. Greater investigative efforts seem necessary in this sense moreover for *Solanaceae,* widely used as food which can be enriched in anthocyanins content with positive effects on human healthy.

bHLH Transcription Factors

Basic helix–loop–helix (bHLH) are TFs present in three eukaryotic kingdoms (Carretero-Paulet et al.,2010). Their mechanism for controlling genes transcription often involve homodimerization or heterodimerization. Typically, a bHLH domain is formed by a stretch of about 18 hydrophilic and basic amino acids at the N-terminal end of the domain, followed by two regions of hydrophobic residues predicted to form amphipathic α-helices separated by an intervening loop (Murre et al. 1994). In plants, the bHLH determine the specificity in recognizing binding sites of target gene promoters and activating transcription (Montefiori et al., 2015). In the model plant *Arabidopsis thaliana*, 133 different bHLH were detected, and at least 113 of them are expressed (Heim et al., 2003). Many bHLH are involved in the anthocyanins pathway regulation and two of them, the *GL3/EGL3* and *TRANSPARENT TESTA8* (*TT8*) are reported to be involved in the MBW complex (Nesi et al. 2001). The *TT8* regulates its own expression as part of a positive feedback loop, through the MBW complex, that ultimately contributes to the regulation of anthocyanin (Baudry et al., 2006; Xu et al., 2013). In Solanaceae, the orthologous of *GL3/EGL3* and *TT8* are identified as *JOHNANDFRANCESCA13* (*JAF13*) and *ANTHOCHYANINS1*(*AN1*), with the exception of *Capsicum annuum* where the identified orthologous of the bHLH involved in the MBW complex is called *CaMYC* (Barchi et al., 2019; Lu et al., 2018). In petunia, both *PhJAF13* and *PhAN1* were identified and was suggested that they could not be mutually exchanged as they are involved in the anthocyanin regulatory cascade in different tissues (Spelt et al., 2000). In tomato, the AN1 protein appears to be involved in anthocyanin

regulations as the overexpression of *SlAN1* greatly elevated anthocyanin content in tomato fruits through the upregulation of *SlF3'5'H* and *SlDFR* (Spelt et al., 2000; Qiu et al., 2016).

In accordance, substantially higher transcript levels of the *CaMYC* in purple pepper have been found and it is also correlated with the upregulation of *F3'5'H* and *DFR* (Liu et al., 2018). In potato, *StAN1*and *StJAF1* results highly express in red and purple tubers, respectively (Payyavula et al., 2013). D'Amelia et al. (2014) found that transformation of either *StAN1* together with *StJAF13* in tobacco resulted in a more intense purple leaves pigmentation than in case of *StAN1* alone. Moreover, AN1 directly activates the anthocyanin biosynthetic pathway through the MYB-AN1-WD40 complex, whereas JAF13 regulates the pathway indirectly, by regulating *AN1* transcription through the MYB-JAF13-WD40 complex upstream in the regulatory cascade (Liu et al., 2018).

WDR Transcription Factors

WD-repeat (WDR) is a component of the MBW complex having a scaffold function for MYB and bHLH proteins. In Arabidopsis, TTG1 interacts with both the bHLH (TT8 and GL3) and the MYB (TT2) to regulate the anthocyanin biosynthesis (Baudry et al., 2004). In the *Solanaceae* the gene encode the WDR proteins are classified as WD40 and the orthologous genes are commonly named *ANTHOCHYANINS11* (*AN11*) or *CaWD40* in pepper. The expression level of WD40 is comparable between anthocyanin-pigmented and non-pigmented tissues (Stommel et al., 2009; Liu Y. et al., 2015; Stommel and Dumm, 2015). Albert et al. (2014) demonstrated that the WDR intercellular movement may facilitate the anthocyanin pigment pattern formation. Moreover, the expression level of tomato *SlAN11* in peel was similar between wild type and anthocyanin-rich *35S::SlANT1* transgenic plants (Kiferle et al., 2015). Consistently to what mentioned above, in many Solanaceous vegetables a basal expression level of WD40 occur regardless anthocyanin is produced or not. In petunia a mutation of *PhAN11* produces a white flowers mutant (Quattrocchio et al., 2006). In pepper the silencing of *CaWD40* by VIGS causes the reduction in transcript levels of structural genes as well as in anthocyanin content (Aguilar-Barragán and Ochoa-Alejo, 2014).

The Regulatory Mechanism Model

A manifold regulatory mechanism is required to a proper biosynthesis of anthocyanins in the correct spatial location and at appropriate levels. The regulatory mechanism acts in both activation and repression of the pathway. During anthocyanins inductive conditions high expression level of the R2R3-MYB activator (ANT1 and/or AN2) are present. The R2R3-MYB activator first interacts with bHLH (JAF13) and WD40 (AN11) forming the R2R3 MYB- JAF13-WD40. This firs MBW complex allows the transcription of a second bHLH (*AN1*). Subsequently, a second MBW activation complex

is formed by the interaction of the same R2R3-MYB activator (ANT1 and/or AN2), the new bHLH (*AN1*) and the WD40 (AN11). This second MBW positively regulates the expression of the LBGs inducing the anthocyanins biosynthesis. On the contrary, during noninductive conditions, low levels of R2R3-MYB activator (ANT1 and/or AN2) are present. Despite the presence of bHLH (JAF13) and WD40 (AN11), which are constitutively expressed, due to the lack of the R2R3-MYB activator the first MBW complex cannot be formed. The lack of the first MBW complex consequently inhibits the transcription of the second bHLH (AN1) which does not allow the formation of the second MBW complex, interrupting the expression of the LBGs and therefore the anthocyanins biosynthesis. Moreover, a repressive fine-tuning systems is involved. Another R2R3-MYB, with a repressor activity, interacts with the bHLH and the WD40 proteins involved in the formation of the first MBW complex, described above. The presence of the R2R3 MYB repressor in the complex (R2R3 repressor-MYB-JAF13-WD40) suppresses the transcription of the second bHLH (*AN1)* producing a repression similar to the one described for the anthocyanins noninductive conditions.

A different mechanism was discovered to explain anthocyanins biosynthesis repressive effect due to the presence of the R3-MYB. The R3-MYB, which contains a single binding domain, is able to bind the bHLH (JAF13) thus sequestering it to the R2R3-MYB activator. The presence of R3-MYB-JAF13 complex inhibit the first MBW complexes formation. Therefore R3-MYB presence dramatically affects the anthocyanins synthesis (Albert et al., 2014; Kroon et al., 2004; Koes et al., 2005).

Anthocyanins in Eggplant: State of the art

QTLs underlying anthocyanin pigmentation

Despite the economic importance of eggplant, genetic mapping and functional studies to disclose the genetic basis of anthocyanin synthesis and accumulation have been rather limited compared to other *Solanaceae* like petunia, tomato and potato. The genetic control of anthocyanin formation, distribution and accumulation has been widely studied in *Solanaceae* species (Van eck et al., 1993, 1994; Chaim et al., 2003; Bovy et al., 2007; Gonzali et al., 2009, Borovsky et al., 2004; De Jong et al., 2004; Stommel et al., 2015). The presence of anthocyanins in eggplants can be considered a quantitative trait, showing a phenotype with a continuous distribution. In eggplant, it was thought for long to be a complex trait, involving several loci with assumed epistatic interactions and/or pleiotropic effects (Tatebe et al., 1939, Tigchelaar et al. 1968).

In the last decade, QTL-related studies employing both bi-parental mapping populations (Barchi et al., 2012; Portis et al., 2014; Toppino et al., 2016) and GWAS approaches (Ge *et al*., 2013; Cericola *et al.,* 2014) allowed to shed light on the genetic bases of anthocyanin distribution in eggplant as well as to identify its syntenic relationships with tomato. The eggplant mapping population obtained by the cross between parental lines '305E40' and '67/3' was used to discover the genetic basis of seven traits associated with anthocyanin content (Barchi et al., 2012) and other key horticultural traits including peel color (Portis et al., 2014) as the two parental lines are divergent both for anthocyanin intensity (67/3 is richer in anthocyanins than 305E40), anthocyanin tonality (violet in 67/3 *versus* reddish in 305E40) and fruit peel color (dark lilac in 67/3 *versus* dark purple in 305E40). Moreover, the same F2 population was used to perform a QTL search for biochemical composition and qualitative traits of fruit, including fruit coloration (Toppino et al. 2016). In particular, a biochemical characterization for both fruit qualitative traits, including dry matter, brix, sugars, and organic acids, as well as for health-related compounds, including chlorogenic acid, the two peel anthocyanins delphinidin-3-rutinoside (D3R) and delphinidin-3-(p-coumaroylrutinoside)-5-glucoside (nasunin) and the two main steroidal glycoalkaloids, (solasonine, and solamargine) was carried out. QTL analysis performed on the bi-parental mapping population allowed to identify two clusters of QTLs, spanning two genomic regions on E05 and E10, associated with anthocyanin content and pigmentation in all eggplant tissues and organs including flowers, vegetative parts and fruit peel and to highlight their synteny with tomato. The high inter-trait correlations between the traits falling in both QTL clusters suggest that they reflect either a set of closely linked loci or, more likely, a single pleiotropic locus.

The QTL cluster identified on chromosome E05, controls fruit color, under calyx color and the content of the anthocyanins D3R and/or Nasunin. The second QTL cluster identified on E10 is associated with the presence of anthocyanins in leaves, stem, and fruit calyx, and may be ascribable to the same QTL previously identified by Doganlar et al. (2002). The QTL clusters located on E5 and E10 are possibly involved in two different aspects of the anthocyanin determination and distribution in eggplant. The QTLs on E05 seem more involved in the alternative production of the nasunin or D3R and it is responsible of the fruit and corolla color. Conversely, the QTLs on chromosome E10 appear mainly involved in the anthocyanin intensity and distribution in all tissues (Zhang et al., 2014 and Docimo et al., 2016).

Recently, the RIL mapping population from the '305E40' x '67/3' cross employed to construct a high density GBS-Based linkage map (Toppino et al., 2020) was phenotyped in three locations for several traits associated to anthocyanins distribution, as the adaxial leaf lamina anthocyanin (*adlan*), corolla color (*corcol*), flower anthocyanin intensity (*flian*), hypocotyl anthocyanin (*hyan*), leaf venation anthocyanin (*lvean*), stem anthocyanin (*stean*), anthocyanin tonality (*toan*) was also submitted to metabolomic analyses of fruits peel and flesh (Sulli et al., 2019). At least one major QTL was spotted for each trait in each location, and the availability of a high-quality map allowed to narrow the confidence interval of each of them with respect to the previous map. The ultra-high density genetic linkage map confirmed that the clusters on chrs. E10 and E05 are involved in the pigmentation of eggplant tissues, which probably are associated to two different aspects of the anthocyanin synthesis among tissues. Indeed, the cluster on chr. E10 is mainly prominent for anthocyanin production and accumulation in the vegetative plant organs. Thanks to the genome annotation available it was finally possible to provide a set of relevant candidate genes involved in the anthocyanin biosynthetic process and in its regulation. The cluster identified on E10 lies in a region of 1.5Mb, between 93.5 and 95 Mb containing three sub-clusters of colocalizing QTLs.

Within the confidence interval, five genes predicted as Anthocyanin Synthase (ANS) were spotted which might be involved in the oxidation of leucocyanidin, along the second to last step of anthocyanin biosynthesis (Saito et al., 1999). A further characterization highlighted that these genes are located in a genomic region of the parental line '67-3' containing retrotransposon like sequences, that could alter the expression pattern of nearby genes (Hirsh et al., 2017). Moreover, a putative MYB transcription factor and a Phosphoinositide phosphatase, belonging to a class of phosphatases that play a key role in abiotic stress response, vacuolar trafficking and anthocyanin accumulation (Williams et al., 2005) were found. Alongside the cluster, an Ankyrin repeat-containing protein reported to be involved in the anthocyanin synthesis pathway (Yoo et al., 2011) was spotted. Finally,

five peroxidases coding genes were identified which may be involved in the degradation of anthocyanin which influence the coloration of the involved tissues. Indeed, enzymatic degradation has been considered to be responsible for anthocyanin breakdown in plants, leading to pigment concentration reduction and color fading (Luo et al., 2019). Conversely, the cluster on chr. E05 contains QTLs more likely associated with the anthocyanin tonality and the type of anthocyanin.

The region identified on chr. E05, which controls stem anthocyanin *(stean),* anthocyanin tonality *(toan),* hypocotyl anthocyanin (*hyan)*and corolla color (*corcol)* contains two slightly separate clusters. The upper region, including the QTLs for *toan5.2* and *stean5.1*, was already spotted by Barchi et al. (2012) as a genomic region involved in the control of several anthocyanin-related traits (as *stean*) and the corolla color. In the same position, Toppino et al. [2018] mapped QTLs associated to peel fruit color as well as to the presence and amount (determined by HPLC) of D3R and Nasunin, the two different anthocyanins in the eggplant peel. Analogous QTLs in the distal portion of E05 were also previously identified by GWAS approaches [Portis et al., 2015].

Anthocyanins related genes and transcription factors

Anthocyanins are highly enriched in eggplants (*Solanum melongena* L.) with purple peel. The varieties of eggplant with dark purple peel, which is caused by a high anthocyanin concentration, are often more attractive to consumers than the paler types.

Higher contents of CGA, Delphinidin 3-rutinoside, and rutin were found in eggplant fruits compared to other tissues, associated to an elevated transcript abundance of structural genes such as PAL, HQT, DFR, and ANS, suggesting that active *in situ* biosynthesis contributes to anthocyanin and CGA accumulation in fruit tissues (Docimo et al., 2018). Although the anthocyanin pathways of synthesis and regulation have been well-studied in *Solanaceous* vegetables, more research is needed regarding eggplant.

In the last decade, several efforts were made from the eggplant-devoted scientific community to fill the gaps of knowledge existing between this species and other Solanaceae about the genetic and environmental regulation of the anthocyanin biosynthetic and degradation pathways, focusing on transcriptomic, biochemical and functional characterization of key genes (Liu et al., 2018). Nowadays studies are elucidating the strong dependence of the fruit peel coloration on tissue, developmental stage and environmental condition (Barchi et al., 2019, Stommel et al., 2015; Docimo et al., 2016, Moglia et al., 2020); however, deeper genetic information about all the eggplant orthologues genes involved in the anthocyanin biosynthesis and modulation are far to be disclosed. Many structural genes encoding keys enzymes related to anthocyanins biosynthesis as *phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarateCoA ligase (4CL), chalcone synthase (CHS),*

chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonoid 3'5'-hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and (3GT) anthocyanidin 3-O-glucosyltransferase were recently well characterized in eggplant (Zhang et al., 2014; Stommel et al., 2015).

Light is a key environmental factor that affects the synthesis of anthocyanins: in a preliminary work, Jiang et al., (2016a) isolated four anthocyanin biosynthesis genes, *SmCHS, SmCHI, SmF3H* and *SmDFR*, and investigated their expression profiles along with other two structural genes (*SmF3'5'H* and *SmANS*) in different tissues, bagging, and low-temperature treatments, revealing the highest expression levels in peels except for *SmF3H* which was detected in stems. Unlike purple peels of the control, the bagged fruits displayed white peels and had no anthocyanin accumulation, because of the expression of *SmCHI*, *SmF3'5'H*, *SmDFR*, and *SmANS* totally depended on light. As low temperature stimulates anthocyanin accumulation, all the six anthocyanin biosynthesis genes were up-regulated in cold stress experiment, and *SmCHS* increased most obviously. In the eggplant peel, the biosynthetic pathway is regulated by light and color becomes evident at most 2 days after exposure to light (Li et al., 2017). This process is facilitated by special photoreceptors which transmit a signal to the downstream transcription factors that regulate plant growth and metabolic processes, including anthocyanin biosynthesis. Downstream of these photoreceptors, Constitutive Photomorphogenic 1 (COP1) acts as a central repressor in the anthocyanin biosynthesis pathway and is considered a 'molecular switch' in metabolic processes which are stimulated by light (Liu et al., 2018). In a recent investigation, the expression patterns of a variety of anthocyanin biosynthesis structural genes and regulator genes period were analyzed aimed at elucidating the molecular mechanisms of light-induced anthocyanin biosynthesis before color becomes evident. transcriptome analysis revealed the presence of 24 anthocyanin biosynthesis structural genes, 102 transcription factors, 3 photoreceptors (*UVR8, CRY3* and *UVR3)* and three light signal transduction elements (*COP1* and two *SPAs*) which exhibited highly dynamic changes in response to light acting as the key regulatory factors in anthocyanin biosynthesis pathway. More recently, He et al., 2019 performed a transcriptome study on bagged pericarp of eggplant to explore the molecular mechanism of dark-regulated anthocyanin synthesis in photosensitive vs non-photosensitive eggplant. Based on the expression trends of the structural genes, it was discovered that 22 transcription factors and 4 light signal transduction elements may be involved in the anthocyanin synthesis in these two types of eggplants. Nine of them were identified to be directly or indirectly bound to the promoter of the structural gene *SmCHS* and participated in the anthocyanin synthesis in eggplant peel forming a network of interactions among themselves.

High temperature is another important environmental factor that may strongly influence the patterns of anthocyanin synthesis and regulation and degradation. An RNA-seq analysis (Zhang et al., 2019)

performed to determine the effects of two different high-temperature stresses (38 °C and 45 °C) on the anthocyanin biosynthetic pathway of eggplant revealed the presence of differentially expressed genes involved in anthocyanin biosynthesis under high temperature stress, including regulatory and structural genes. These results indicated that high temperature is able to downregulate most of the genes in the anthocyanin biosynthetic pathway of eggplant.

Anthocyanin accumulation is determined by the balance between biosynthesis and degradation. In the daily diet, the eggplant fruits are usually consumed after cooking, steamed to spiced dishes, boiled, steamed, fried, etc. Anthocyanins are unstable and easily degraded, especially under thermal treatments, and when anthocyanin degradation occurs, polymeric compounds might be also produced (Lo Scalzo et al., 2016). Anthocyanin degradation is affected by several endogenous and exogenous factors, such as phenolics, protein, carbohydrate, light, temperature, oxygen, pH, metal ions, and so on. Zhang et al., (2020) identified the pathway of anthocyanin degradation by exploring the effects of food status and thermal treatment on degradation of crude anthocyanins from eggplant peel, formation of brown/polymeric color and changes of antioxidant capacity. Despite the knowledge regarding this argument is growing, anthocyanin pathway is still largely unknown in eggplant.

MBW transcriptional complex in eggplant (*S. melongena* L.)

Eggplant berry's peel has been recognized as very rich in anthocyanins (Mennella et al, 2012) at the same time the growing availability of genomic resources, makes it an exploitable species to better clarify the mechanisms underlying the accumulation of anthocyanins in the *Solanaceae*. Among the factors affecting the accumulation of anthocyanins in eggplant, the regulation of the pathway by TFs was deeply investigated. It is known that the over expression of the *SmMYB1* (R2R3MYB) produce a dramatical up-regulation of the structural genes *SmCHS, SmF3H, SmF3'5'H, SmDFR, SmANS,* and *SmUFGT* (Zhang et al. 2016). More recently, the putative orthologs of the two CGA biosynthetic genes *PAL* and *HQT*, as well as a variant of a *MYB1* transcription factor showing identity with group six MYBs, were isolated from an Occidental *S. melongena* traditional variety and demonstrated to differ from published sequences from Asiatic varieties. *In silico* analysis of the isolated *SmPAL1, SmHQT1, SmANS*, and *SmMyb1* promoters revealed the presence of several Myb regulatory elements for the biosynthetic genes and unique elements for the TF, suggesting its involvement in other physiological roles beside phenylpropanoid biosynthesis regulation. Transient overexpression in *Nicotiana benthamiana* leaves of *SmMyb1* and of a *C-terminal SmMyb*1 truncated form (*SmMyb1Δ9*) resulted in anthocyanin accumulation only of *SmMyb1* agro-infiltrated leaves suggesting that a deletion of the C-terminal region of *SmMyb1* does not limit its capability to regulate CGA accumulation but impairs anthocyanin biosynthesis. The *SmMYB1* and *SmMybC* displayed higher

transcript levels in anthocyanin-pigmented fruits compared to non-pigmented fruits (Zhang et al., 2014b; Stommel and Dumm, 2015; Gisbert et al., 2016). The significant upregulation of *SmMYBs* was in accordance with the elevated expression level of structural genes and anthocyanin content. Xi et al., (2020) found that SmbHLH13 bonded and activated the expression of the structural genes *SmCHS* and *SmF3H* in anthocyanin biosynthesis and also to the promoter of the key gene *SmFT* during flowering. Furthermore, genetic transformation of *Arabidopsis* revealed that overexpression of *SmbHLH13* enhanced anthocyanin accumulation and delayed flowering, thus demonstrating that *SmbHLH13* might promote anthocyanin accumulation through positive regulation of *SmCHS* and *SmF3H*. The spatial and temporal expression of the LBGs is determined by a combination and interactions of different families of TFs. Interestingly, due to their tissue specificity, it can be hypothesized that different MBW complexes may act to promote anthocyanin synthesis by recruiting *ANT1* and *AN2* in the fruits and in flowers, respectively (Kiferle et al., 2015). Therefore, the orthologous of tomato *ANT1* and *AN2* as well as of the eggplant orthologs of tomato *AN1* and *JAF13*, two bHLH factors, known to be involved in the MBW complex, have been identified (Cericola et al., 2014; Docimo et al., 2016; Barchi et al. 2019). The expression profile of both *SmAN1* and *SmJAF13* resembles the one of *SmANT1* and *SmAN2*, as well as of *DFR* and *ANS*, thus corroborating the hypothesis that they play a role in the MBW regulatory complex. Differently to other *Solaneceae,* in eggplant the involvement of other elements of the MBW complex, such as the AN11 *(*WD40) and some MYBs (R2R3MYB and R3MYB) with repressive functions of the MBW complex have not been yet demonstrated.

Anthocyanins “Decoration” related genes

In contrast to the large knowledge available to the structural genes related to the anthocyanin's pathway of eggplant, the genes involved in the anthocyanin's “decoration” are still slightly characterized. Recently, QTL analysis performed on the bi-parental mapping population allowed to identify two clusters of QTLs associated with anthocyanin pigmentation, one on chromosome E05 and the other on E10. The QTLs on E05 seem more involved in the alternative production of the nasunin or D3R and it is responsible of the fruit and corolla color. Anthocyanin related QTL’s together with the recent eggplant genome sequence of 67/3 (Barchi et al., 2019) are actually the key tools to better understand the process underling anthocyanins biosynthesis in eggplant. Recently, the deep characterization of the QTLs associated to peel fruit color as well as to the presence and amount of D3R and Nasunin performed by Toppino et al. (2020) in a RIL population from the cross 305E40 x 67/3 highlighted the presence of a *Acetyl-CoA-benzylalcohol acetyltransferase* (*AAT*) eligible as the best gene candidate for the conversion of D3R into nasunin. The comparison of the Illumina

sequencing data available for the two parental lines 67/3 and 305E40, characterized by the presence of Nasunin and D3R, respectively, revealed a SNP which could determine a loss-of-function mutation in the *305E40_AAT* CDS sequence, opening the path to a deeper functional study of this gene.

Characterization of eggplant berries phenols across ripening stages

Eggplant composition is affected by several factors, such as environmental conditions, growing systems, genetic background and ripening stage. To the best of our knowledge, there is a lack of knowledge over the phenols composition for eggplant berries ripening stages. Fruited species are generally classified into two groups: climacteric and non-climacteric, according to presence or not of a peak in respiration and ethylene production during ripening (Lelièvre et al., 1997). Non-climacteric fruit may respond to ethylene, but it is not required for the complete ripening in contrast to the climacteric ones where the ethylene is required (Giovannoni, 2001). Conversely to what happens in the other major *Solanaceous* fruit crops like tomato and pepper, the commercial ripening stage of the eggplant berry is always extremely anticipated with respect to the physiological ripening stage, which represents a commercially waste product. Tomato and eggplant differ with respect to their ethylene dependence: tomatoes are climacteric fruits where ripening is under the control of ethylene, while eggplant is a non-climacteric fruit where ethylene appears to have little influence on ripening (Kader, 1992). Although it was recently found that the ethylene signaling inhibitor 1-methylcyclopropene (1-MCP) was able to delay senescence and maintain postharvest eggplant fruit quality (Massolo et al., 2011). Therefore, the process of ripening in eggplant is less understood and few studies have been performed mainly focused on the accumulation of the anthocyanin pigment, sugars, phenolics and glycoalkaloid compounds (Esteban et al., 1992; Mennella et al., 2012). Three main ripening stages during eggplant berries development are generally examined: unripe (stage A), commercially ripe (stage B) and physiologically ripe (stage C). Fruits at the Stage A and B show a pigmented and glossy peel, firmer flesh and harder skin and the seeds are not or little visible with respect to the Stage C which is characterized by the presence of mature seeds in the fruit, together with a loss of firmness in the flesh and a deep pigmentation change of the skin, (which turns from the typical commercial colors to a wide range of browns, greys or yellows when fully physiologically ripe), and a loss of glossiness and brightness that can be genotype-dependent. In the physiologically ripen fruit, the change of color and glossiness of the skin deeply affects the commercial value of the product, which are unmarketable. For this reason, the fidelity of the berry pigmentation to the right commercial typology and its durability along the ripening are among the key traits of interest considered in the breeding programs aimed at the improvement of the commercial value of the eggplant fruit and to the new varietal constitution. However, this latter ripening stage has been largely ignored in eggplants

but the similarities and differences between the process tomato and eggplant therefore make these species very interesting to study and compare fruit ripening processes and regulation. Besides it is known that during the ripening process there is a great remodulation in the anthocyanin content of the eggplant berries (Mennella et al., 2012). It is also known that anthocyanin structural genes and regulators share a marked down-regulation following the ripening stages (Barchi et al., 2019). In spite of this, the knowledge on chemicals product accumulated at stage C and the molecular basis of the anthocyanins remodeling during the ripening of eggplant are still lacking and poorly reported.

Thesis objectives

The overall purpose of this Ph.D. research project is the molecular characterization of a panel of genes involved in the biosynthetic pathway of anthocyanins in eggplant (*S. melongena* L.) and in their regulation among different accessions or across the ripening stages of the fruit.
The anthocyanin biosynthetic pathway has been characterized in different *Solanaceous* species like tomato, pepper, eggplant, petunia, tobacco and potato. Despite the knowledge regarding this argument is growing, anthocyanin metabolism is still largely unknown in eggplant. Anthocyanins pathway is very intricate because it appears finely regulated at molecular/developmental levels and affected by environmental conditions.

The general objectives proposed in this work are:

1) A better clarification of the regulatory genetic basis of the anthocyanin biosynthetic pathway among the *Solanaceae* through the characterization in eggplant of the members of the MBW complex in different tissues, including the recently identified category of MYB repressors, taking advantage of the most modern genomic resources available as the recently release of a new draft of the Eggplant Genome (Barchi et al., 2019) and the synteny that exist between our specie and the model species *S. lycopersicum* and *Petunia* spp.
2) A deep characterization of the QTLs already spotted in the F2 and RIL populations from the cross 305E40 x 67/3 associated with the anthocyanin type, the identification of a putative candidate gene for the conversion between D3R into nasunin, its validation through a functional genetics approach and the characterization of its allelic variants in a wide germplasm collection of eggplant.
3) The investigation about the modulation of expression profiling of some anthocyanin genes associated to the biochemical composition changes during ripening stages especially focused on the physiologically mature eggplant fruit.

Chapter 1: Identification of a new R3 MYB type repressor and functional characterization of the members of the MBW transcriptional complex involved in anthocyanin biosynthesis in eggplant (*S. melongena* L.)

Abstract

Here we focus on the highly conserved MYB-bHLH-WD repeat (MBW) transcriptional complex model in eggplant, which is pivotal in the transcriptional regulation of the anthocyanin biosynthetic pathway. Through a genome-wide approach performed on the recently released Eggplant Genome (cv. 67/3) previously identified, and reconfirmed by us, mem- bers belonging to the MBW complex (*SmelANT1, SmelAN2, SmelJAF13, SmelAN1*) were functionally characterized. Furthermore, a regulatory R3 MYB type repressor (*Smel-MYBL1*), never reported before, was identified and characterized as well. Through a RT-qPCR approach, we revealed specific transcriptional patterns of candidate genes in different plant tissue/organs at two stages of fruit development. Two strategies were adopted for investigating the interactions of bHLH partners (*SmelAN1, SmelJAF13*) with MYB counterparts (*SmelANT1, SmelAN2* and *SmelMYBL1*): Yeast Two Hybrid (Y2H) and Bimolecular Fluorescent Complementation (BiFC) in *A. thaliana* mesophylls protoplast. Agroinfiltration experiments highlighted that *N. benthamiana* leaves transiently expressing *SmelANT1* and *SmelAN2* showed an anthocyanin-pigmented phenotype, while their coexpression with *SmelMYBL1* prevented anthocyanin accumulation. Our results suggest that SmelMYBL1 may inhibits the MBW complex via the competition with MYB activators for bHLH binding site, although this hypothesis requires further elucidation.

Introduction

Anthocyanins are the major plant flavonoid compounds, which confer appealing colors to flowers and fruits and contribute to stress tolerance (D'Amelia et al., 2018; Kovinich et al., 2015).,In plant vegetative tissues, anthocyanins play key roles in protection against UV radiation, low/high temperatures, drought and pathogen attacks, while in reproductive organs they exert also an eco-physiological role by attracting pollinators and seed dispersers. Anthocyanins also possess widely documented antioxidant, antidiabetic, antihyperlipidemic, anti-inflammatory, anticarcinogenic properties and a preventive activity against cardiovascular diseases in humans (Putta et al., 2017). Eggplant purple fruits are a rich source of anthocyanins, being delphinidin-3-*p*-coumaroyl-rutinoside-5-glucoside and the delphinidin-3-rutinoside the most abundant (Mennella et al.,l 2012) . The anthocyanin pathway represents one branch of flavonoid metabolism and it is a very conserved network in many plant species, with most of the genes encoding for enzymes and regulatory

transcription factors (TFs) identified in several plant species (Albert et al., 2014). The anthocyanin pathway is one of the most finely tuned and it is under the control of Early (EBGs) and Late (LBGs) Bio- synthetic Genes in dicotyledonous species (Liu et al., 2018; Li et al., 2014). *Chalcone synthase* (*CHS)*, *Chalcone-flavonone isomerase* (*CHI)*, *Flavanone 3-hydroxylase* (*F3H)* and *Flavonol synthase* (*FLS)* are common EBGs involved in the biosynthesis of all downstream flavonoids, whose enzymatic steps are controlled by co-activator independent and functionally redundant 'R2R3-type MYB' regulatory genes (*MYB11*, *MYB12*, *MYB111*) (Petroni et al., 2011). *Flavonoid 3' hydroxylase* (*F3'H)*, *Flavonoid-3',5'-hydroxylase* (*F3*^{0}*5*^{0}*H)*, *Dihydroflavonol 4-reductase* (*DFR)*, Anthocyanidin synthase (*ANS*) are LBGs required for anthocyanin synthesis and modification, and their correlation with anthocyanin content has been highlighted in many *Solanaceous* species including eggplant [Liu et al., 2018; Zhang et al., 2014;Li et al., 2018),. RNAseq analyses showed that most of the eggplant LBGs were up-regulated in flower and young fruit skin tissues at the early stage fruit development of ripening, with a marked decrease at the physiological stage of ripening Barchi et al., 2019). It has been reported that the activation of LBGs is mediated by the MYB-bHLH-WD40 (MBW) transcription complexes. MYB proteins, together with bHLH and WD40, can act as positive or negative transcriptional regulators binding to the promoters of structural genes. The MYB family is one of the largest in flowering plants, with 125 members in *A*. *thaliana* (Stracke et al., 2001) sharing common features at amino terminus: the DNA binding domain consists of one to three conserved HLH motifs, referred as R1, R2, R3. The MYB activators mainly belong to the R2R3-MYB family of transcription factors, while repressors consist of both R2R3-MYB and R3-MYB. It has been proposed the involvement of diverse MBW complexes depending on the occurrence of activator or repressor MYBs which directly, and competitively, bind the bHLH *via* the amino terminus domain, acting in a tissue-specific mode to modulate anthocyanin synthesis (Ramsay et al., 2015). Indeed, the MBW complex is counterbalanced by the amount of MYB repressor(s) which inactivate the complex by recruiting the bHLH partner (Albert et al., 2014. The WD40 proteins (*e.g*. PhAN11) modulate the activity of MYB/bHLH regulators at post-transcriptional level and there are no evidences of a direct interaction with these TFs (De Vetten et al., 1997). In the *Solanaceae*, the genes encoding R2R3-MYB transcription factors are orthologs of the petunia *PhAN2 (*Qurattrocchio et al., 1999*)*, while those encoding IIIf group of bHLHs are orthologs of the two petunia groups: the *AN1* and the *JAF13* (Spelt et al., 2000; Kiferle et al., 2015); the physical interaction of PhAN1 and PhAN2 proteins is required to activate the transcription of LBGs (*e.g*. *DFR*) (Spelt et al., 2000). In eggplant, the orthologs of tomato *ANT1* and *AN2* (Kiferle et al., 2015)], belonging to R2R3-MYBs, have been identified and found to be preferentially expressed in the early fruit maturation stage as well as in flowers (Barchi et al., 2019) The transient expression of *SmelANT1* in tobacco (Barchi et al., 2019;

Docimo et al., 2016) and the stable expression into a non-anthocyanin accumulating eggplant (Zhang et al., 2014; Docimo et al., 2016; Zhang et al., 2016) both lead to anthocyanin synthesis, suggesting its role in controlling fruits and flower pigmentation. The orthologs of tomato *AN1* and *JAF13* belonging to bHLH family have been identified in eggplant as well (Barchi et al., 2019) . The role of MYB repressors has only recently being recognized thanks to the studies in petunia, grapevine, poplar and tomato (Cavallini et al., 2015; Colanero et al., 2018). The R2R3-MYBs can act as both positive and negative transcriptional regulators. Indeed, the petunia R2R3-type *Ph*MYB27 acts as repressor of anthocyanin biosynthetic pathway, since its overexpression leads to a reduced anthocyanin content (Albert et al., 2014; Ma et al., 2019). Recently, a R3-MYB encoding gene with three DNA-binding domain repeats, namely *ATROVIOLACEA* (*ATV*), has been characterized in tomato (Colanero et al., 2018); however, genes encoding MYB repressors have not been yet identified in eggplant, pepper, and potato. Recently a high quality, annotated and anchored eggplant genome sequence (www. eggplantgenome.org; Barchi et al., 2019) has been made available, leading to the identification of genes of MBW complex (*Smel*ANT1, *Smel*AN2, *Smel*JAF13 and *Smel*AN1), in the present paper we confirmed their sequence homology also by functional domain identification and phylogenetic analyses. Furthermore, we identified, for the first time in eggplant, a regulatory R3 MYB repressor according to its high similarity with MYBL1, recently described in the genus Iochroma (*Solanaceae*) by Gates and co-workers (Gates et al. 2018). The expression dynamics of candidate genes were assessed at two stages of fruit development and in flowers. To the best of our knowledge, this is the first report on the establishment of eggplant interaction of MYB proteins with bHLH partners, which we assessed both in a yeast two-hybrid system and *via* Bimolecular Fluorescent Complementation (BiFC) in *A. thaliana* mesophyll protoplasts. At last, we estimated the effect of over-expression of the candidate genes in agroinfiltrated *N. benthamiana* plants and established their effect on anthocyanin content.

Materials and Methods

Plant material and growth conditions

Plants of *S. melongena* line "67/3" with violet-black round fruits, were grown in pots (30 cm diameter) in glasshouse at CREA (Montanaso Lombardo, Lodi-Italy), under standard conditions, from March to September 2017. For each organ (open flowers, fruits), samples were obtained by pooling tissues collected from at least 3 plants. At least one flower per plant was collected at anthesis. Skin and flesh of the fruits were collected at the unripe (stage A) and commercial ripening stage (stage B) (Fig. 1.1) according to Mennella et al. (2012). All fresh tissues were immediately frozen in liquid nitrogen and stored at -80℃.

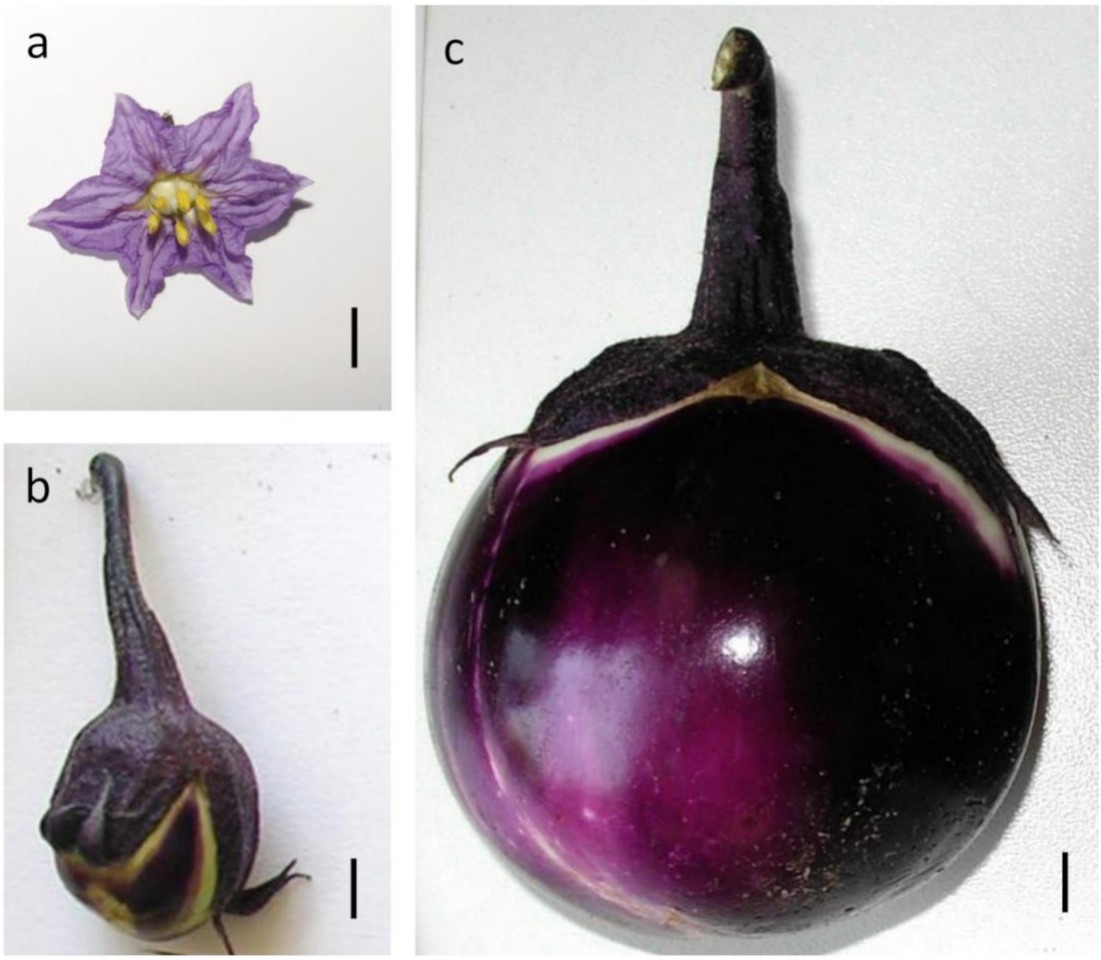

Figure 1.1: Stages of the eggplant flower and fruit employed: A) Open flowers; B) Fruits Ø 2–4 cm at 8–14 DAF (named stage A); C) Fruits at commercial ripening (named stage B) at approximately 38 DAF. Scale bar in each image represents 1cm.

Phylogenetic analysis and identification of regulatory protein

The deduced amino acid sequences of bHLHs and MYBs were obtained by screening the genome of the eggplant breeding line 67/3 (Docimo et al., 2016) using the pBLAST function tool of Eggplant Genome Browser (http://www.eggplantgenome.org/), with the amino acid sequences of petunia and tomato regulatory anthocyanin proteins as query (supplementary files S1 and S2 of Moglia et al. 2020). Sequence alignment of bHLH and MYBs from eggplant as well as from known anthocyanin related bHLH and MYB in other species (from NCBI and Sol Genomic Network (https://solgenomics.net) were generated via multiple sequence alignment using the ClustalW algorithm in the MEGA X package (Kumar et al.2018). The evolutionary history of both families of TFs was

visualized by the Neighbour-Joining method via MEGA X. All ambiguous positions were removed for each sequence pair (pairwise deletion option) resulting in 1301 (for bHLH tree) and 2561 (for MYB tree) positions in the final dataset. The statistical significance of individual nodes was assessed by bootstrap analysis with 1,000 replicates, and the evolutionary distances were calculated using the p-distance method with default parameters. A second round of alignment and phylogenetic analysis was performed, as described above, with a limited number of MYBs belonging to known flavonoids repressor proteins.

MYBL1 structure analysis

The nucleotide sequence data of *SmelMYBL1* (*SMEL*_010g336390.1) is available at GeneBank Database with accession number MN855525. The Intron/Exon organization for *SmelMYBL1* gene was determined by aligning the cDNA sequences to their corresponding genomic DNA sequences used as the input for graphical display at the Gene Structure Display Server of Peking University, China (http://gsds.cbi.pku.edu.cn/). Moreover, the protein sequence of *Smel*MYBL1 was aligned to other similar MYBL1 proteins using ClustalW in MEGA software.

Real-time PCR analysis

Total RNA from eggplant cv. 67/3 tissues was extracted using the TRIzol RNA Isolation Reagents (Thermo Fisher Scientific) combined with the Spectrum Plant Total RNA kit (Sigma Aldrich). The single strand cDNA was synthesized from 1 µg of RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, USA). Amplifications were performed with primers designed by Primer 3 software (https://bioinfo.ut.ee/primer3-0.4.0/primer3/) for eggplant *AN2*, *ANT1*, *AN11*, *JAF13*, *AN1*, *DFR*, *MYBL1*. All the gene-specific primer sequences are listed in Table1.1. A standard amplification curve was generated for each gene using 2-fold serial dilution of pooled cDNA. PCR efficiency was optimized to be in the range 80–100% with R^2-values of 0.996. The PCR reactions were carried out using the Rotor-Gene RG-6000 thermal cycler (Corbett Research) according to the following PCR parameters 95℃ for 5 min, followed by incubation for 15s at 95℃ and denaturation for 15 s at 95℃, annealing for 60 s at 59℃ for 40 cycles, followed by elongation at 72℃ for 20 s. Specificity of amplifications was assessed by melt curves analyzed for the presence of a single peak. The analyses were performed on three biological replicates and in technical triplicates. Expression of *Smel*GAPDH (Barbierato et al. 2017) was used as reference gene. Relative expression levels of each individual gene were calculated using GeNorm (https://genorm.cmgg.be/)(Vandesompele et al., 2002).

Table 1.1: List of primers used in this chapter.

Gene	Sequences	Scope
SmelANT1	FW 5'- ggaccgcaaacgatgtaaag-3'	RT-qPCR
	RV 5'- tttccgaggttgaggtcttatt-3'	
SmelAN2	FW 5'-aacgccacaaagaaattgga-3'	RT-qPCR
	RV 5'-ccagcaaacttgtccaccat-3'	
SmelAN1	FW 5'- ttgcccagacggtagaac-3'	RT-qPCR
	RV 5'- cagccgacccaaccccactt-3'	
SmelJAF13	FW 5'-actgttcaggcaggggaagt-3'	RT-qPCR
	RV 5'-cctgtggttgtatgtcgtct-3'	
SmelAN11	FW 5'-actgtgccccttgacctct-3'	RT-qPCR
	RV 5'-attgggtttcgacagcccc-3'	
SmelMYBL1	FW 5'-ccttggtgaaagatggtcgc-3'	RT-qPCR
	RV 5'-ggaactttcagcgtcagaagc -3'	
SmelDFR	FW 5'-atacaaacttaacgctgtggaaag-3'	RT-qPCR
	RV 5'-agtcgctccagctagtctcgtca-3'	
SmelGAPDH	FW 5'- ggtgccaagaaggttgtgat-3'	RT-qPCR
	RV 5'- ccaatgctagttgcacaacg-3'	
SmelAN2	FW 5'-caccatgaatactgctactgttgctaagtca-3'	Gateway cloning
	RV 5'-cccctaattaaatagattccataggtca-3'	
SmelMYBL1	FW 5'-caccatgagcaaggataaaatacaag-3'	Gateway cloning
	RV 5'-ttaatgtgcagaggaactttcag-3'	
SmelANT1	FW 5'ggggacaagtttgtacaaaaaagcaggctcgatgaataatcctcctataatctgtacgtctg-3'	Gateway cloning
	RV 5' ggggaccactttgtacaagaaagctgggtccttaatcaagtaaattccataaatcaatatca-3'	
SmelJAF13	FV 5'- ggggacaagtttgtacaaaaaagcaggctcgatggctatgggacaccaag-3'	Gateway cloning
	RV 5'- ggggaccactttgtacaagaaagctgggtcctcaagatttccaaactgctctc-3'	
SmelAN1	FV 5'- ggggacaagtttgtacaaaaaagcaggctcgatgatggagatcatacagcctag-3'	Gateway cloning
	RV 5'- ggggaccactttgtacaagaaagctgggtccttaaactctagggattatctgatgt-3'	

Cloning of the MYBs and bHLHs encoding genes

*Smel*ANT1, *Smel*AN2, *Smel*MYBL1, *Smel*JAF13, *Smel*AN1 were first amplified from cDNA using primers with attB1 and attB2 sites, cloned by Gateway Recombinant Technology in pDONOR 207 vector through a BP recombination and subsequently transferred by LR recombination into the destination vector, as described in the following paragraphs.

Yeast two-hybrid

ProQuestTM Two-Hybrid System (Life Technologies), kindly provided by Dr. Montanini from University of Parma, was used. Each entry vector (pDONOR207 with the CDS of the gene of interest as detailed above) was recombined with the activation domain (AD) vector pDEST22 (for ANT1, AN2 and MYBL1) and/or the binding-domain (BD) vector pDEST32 (for JAF13 and AN1). *S. cerevisiae* strain Mav203 was transformed with 1µg each of the different combi- nations of bait, prey and control (non-recombined) vectors using the lithium acetate/polyethylene glycol method. Transformed colonies containing bait and prey plasmids were selected on synthetic dropout medium lacking Tryptophan and Leucine (−W/−L). To test the interaction between bait and prey, an equal number of cells was spotted on medium lacking Tryptophan, Leucine and Uracil (−W/−L/−U). Negative controls using empty vectors were also performed. Two round-shaped 50 mm Whatman 541 filter papers, saturated with 3.5 ml of 2% X-gal solution, were placed on a 10 cm petri dish. The transformed yeasts from the surface of the YPAD plates were obtained by a 50 mm Whatman 541 filter paper and then completely immersed in liquid nitrogen for 15 seconds and then set them on the top of the soaked Whatman filters. Plates were sealed with a parafilm and incubated at 37℃ for overnight. The results were recorded after 1 hours by photography.

Bimolecular Fluorescent Complementation (BiFC) analysis

A. thaliana Columbia-0 ecotype mesophyll protoplasts were isolated and transformed as previously described (Iacopino et al., 2019). AN1 full CDS entry vector was recombined with N9842 vector (Zhong et al., 2008) to generate AN1-nYFP fusion protein. AN2, ANT1 and MYBL1 full CDS were instead recombined with the N9843 vector (Zhong et al., 2008) to generate AN2-cYFP, ANT11-cYFP and MYBL1-cYFP fusion proteins, respectively. Both N9842 and N9843 vectors were kindly provided by Dr. Beatrice Giuntoli (Department of Biology, University of Pisa). As negative control, plasmids containing the expression cassette for nYFP-GUS or cYFP-GUS fusion proteins (Shukla et al. 2019) were used in combination with the previously described vectors. Plasmid DNA was isolated using a DNA Maxi-prep kit. Protoplasts were transformed using 2.5 µg of each plasmid and stained with 2 µg of DAPI (Sigma-Aldrich). Confocal investigation was performed with the Zeiss AiryScan confocal microscope. YFP fluorescent was exited with a 488 nm laser and collected at 490–540 nm. Chlorophyll fluorescent was exited with 640 nm laser and collected at 650–750 nm. DAPI was exited at 405 nm and collected at 410–470 nm. Images were analysed with the ZEN 2010software (Zeiss).

Transient heterologous expression in *N. benthamiana*

For transient expression, the pEAQ-HT vector kindly provided by Prof. Lomonossoff (Sainsbury et al., 2009), was used. Each entry vector (pDONOR207 with the CDS of the gene of interest as detailed above) was then recombined with the pEAQ-HT destination vector. The pEAQ-HT destination vectors (containing genes of interest) as well as the empty vector pEAQ-HT, used as a negative control, were inserted in *Agrobacterium tumefaciens* strain C5801 by the freeze-thaw method. Transformed bacteria were grown overnight at 28℃ in 5 mL of L medium (10g L^{-1} bactotryptone, 5 g L^{-1} Yeast extract, 5 g L^{-1} NaCl, 1 g L^{-1} D-glucose) containing kanamycin (50 mg L^{-1}). The overnight cultures (2 mL) were then transferred into 20 mL of induction medium (L broth containing 10 mM MES and 20 μM acetosyringone) with kanamycin (50 mg L^{-1}) and grown as above. The cells were collected by centrifugation for 10 min at 4,000 g and resus- pended in 50 mL of infiltration medium (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone) to an OD_{600} of 1.0 and kept at room temperature for 3 h before being infiltrated into the abaxial air spaces of 2–4-week-old *N. benthamiana* plants. After 4 days, the infiltrated leaf material was collected and used for quantitative HPLC/PDA analysis as described below.

Identification and quantification of anthocyanins

Transiently transformed *N. benthamiana* grinded tissues (1 g) were extracted with 8 mL EtOH/HCl (85/15), pH = 1. After centrifugation (10,000 g for 10 min), supernatants were obtained. The identification and quantification of delphinidin 3-*O*-rutinoside was carried out by HPLC on a Shimadzu XR system equipped with a photodiode detector SPD-M20A (Shimadzu, Dusseldorf Germany). HPLC-grade acetonitrile (HPLC plus 99.9%) and formic acid (> 98% purity) were purchased from Sigma Aldrich (Bellefonte, USA). De-ionized water (18.2MO cm) was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Delphinidin 3-*O*-rutinoside was obtained from Extrasynthese (Genay Cedex, France). Each acid extract prepared was filtered with a 13 mm diameter, 0.22 μm pore diameter hydrophilic PTFE syringe filter and then analyzed on an Ascentis Express C18 column (15 cm ×2.1 mm, 2.7 μm, Supelco, Bellefonte, USA) using water/formic acid (99:1, v/v) and acetonitrile/formic acid (99:1, v/v) as mobile phases A and B, respectively. The flow rate was 0.4 mL min^{-1} and the column temperature was maintained at 30℃. The gradient program was as follows: 5% B for 15 min, 5–20% B in 5 min, 20–100% B in 6 min, 100% B for 2 min. Total pre- running and post-running time was 36 min. UV spectra were acquired over the 220–700 nm wavelength range. The quantification of delphinidin 3-*O*-rutinoside (Retention Time: 19.155 min) in the extracts was performed through the external calibration method at 520 nm.

The calibration curve (Figure1.2) of the authentic commercial standard was prepared with six different concentrations, in the 50–1 μg mL^{-1} range (curve equation: y = 14200x-13960, R^2 = 0.9996). All the data were statistically analyzed using SPSS statistical software.

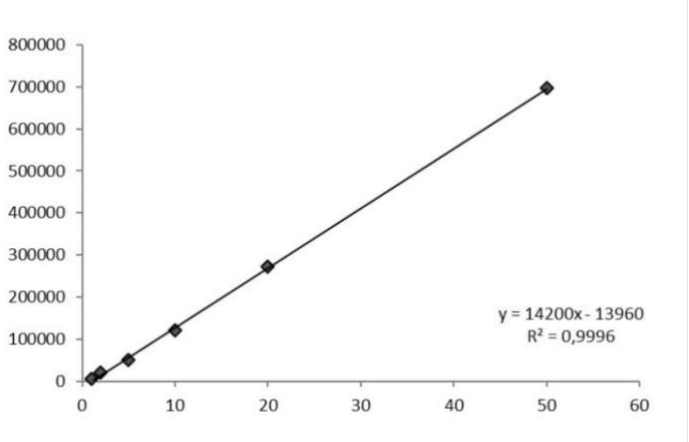

Figure1.2: Calibration curve of delphinidin 3-O-rutinoside standard (50, 20, 10, 5, 2, 1 μg/mL).

Results and discussion

Identification of eggplant anthocyanin-related regulatory genes

Based on a genome-wide phylogenetic approach, we identified eggplant putative MYB and bHLH transcription factors as candidates for anthocyanin regulation. The bHLH encoding genes were spotted by screening the genome of *S. melongena* cv. 67/3 (Barchi et al., 2019) with 13 known plant proteins related to anthocyanin synthesis belonging to the subgroup IIIf (Davies et al., 2012), whose members are known to be involved in flavonoid regulations. A total of 84 gene sequences encoding for bHLHs were retrieved in the eggplant genome and manually inspected, while 159 and 124 were previously identified in tomato (Sun et al., 2015) and potato (Wang et al., 2018) genomes respectively. The eggplant bHLHs were then used to construct a phylogenetic tree based on the NJ method (Fig.1.3) together with 13 known plant proteins related to anthocyanin synthesis belonging to the subgroup IIIf. Among eggplant proteins, two bHLH factors, i.e. *SMEL*008g319200.1 and the *SMEL*009g326640.1 showed high homology with the major plant bHLH factors belonging to subgroup IIIf. These sequences were previously identified by Barchi et al. (2019) and named *SmelJAF13* and *SmelAN*1 in accordance with their homology with tomato Sl*JAF1*3 and Sl*AN1*, respectively. The two proteins fall into distinct evolutionary sub-clades of bHLH involved in anthocyanin regulation of which one comprises members such as JAF13 (tomato, eggplant, petunia and related sequences of other species) and GL3/EGL3 from *A. thaliana*, while the other includes

members such as AN1 (tomato, eggplant, petunia, and related sequences in other species) and TT8 from *A. thaliana* (Fig.1.3). The Zm*IN1* gene has been considered as a separate group by several authors, due to its unique intron-exon structure (Spelt et al., 2000). However, no further studies have been performed to elucidate this putative evolutionary divergence. Depending on the species, the two bHLHs are not functionally redundant and they do not complement each other. Indeed, they might regulate anthocyanins synthesis in a specific time and space manner. In tomato and petunia, both JAF13 and AN1 proteins appear to be involved in anthocyanin regulations although in a different manner (Spelt et al., 2000; Kiferle et al., 2015). A similar approach was applied to identify eggplant anthocyanin related MYBs protein encoding genes. The latter were identified by screening the genome of *S. melongena* breeding line 67/3 (Barchi et al., 2019) with 25 known plant MYBs related to anthocyanin synthesis belonging to subgroup 4–7 according to the classification by (Liu et al., 2015). Interestingly, an analogous number of MYB proteins, *i.e.* 127, were identified in tomato (Li et al., 2016) while 159, were detected in potato (Sun et al., 2019).

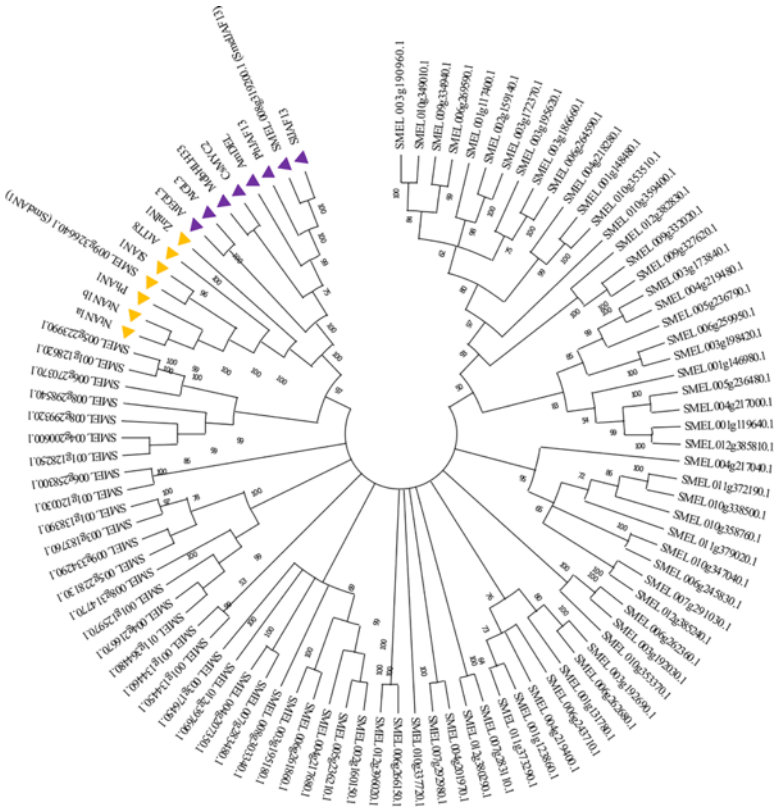

Figure 1.3 ***Phylogenetic tree of bHLH transcription factors in S. melongena genome.*** *The optimal NJ tree with the sum of branch length = 31.39844401 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. This analysis involved 97 amino acid sequences, 84 from Eggplant genome (Barchi et al.,2019) and 13 from known anthocyanin related bHLH in other species. The AN1 and the JAF13 clade described in the text are marked with orange and purple triangle, respectively.*

The identified eggplant MYBs, as well as 25 related MYBs detected in other plant species, were used to construct a phylogenetic tree using the NJ method together (Fig 1.4). Based on similarity with the R2R3-MYB proteins PhAN2, SlAN2 and SlANT1, which are known to be involved in anthocyanin regulation in petunia and tomato, two homologs were identified in eggplant: *SMEL*010g351850.1, corresponding to *Smel*AN2, and *SMEL*001g120500.1 ortholog to *Smel*ANT1, both of which were previously annotated as putative candidate regulatory MYB in eggplant (Barchi et al.,2019; Docimo et al., 2016). Moreover, a number of MYB domain encoding proteins were found in evolutionary subgroups closely related to the ones of known anthocyanins regulators. Interestingly, a cluster of 7 R2R3-MYBs, including *SMEL*007g274170.1 and its paralogous genes *SMEL*007g274160.1, *SMEL*001g153180.1, *SMEL*005g234040.1, *SMEL*008g299860.1, *SMEL*008g299200.1 and *SMEL*008g318650.1, might represent other flavonoids regulatory proteins. Several putative repressors of anthocyanin biosynthesis were identified in eggplant. As depicted in Figs 1.4 and 1.5A, we identified three clades corresponding to different repressor types: (i) a clade including CPC of three repeat binding domain R3 repressors, which comprises *A. thaliana* TRY and CPC, tomato ATV (Colanero et al., 2018; Cao et al., 2017) and TRY, the putative *S. melongena* ortholog of TRY, *Smel*001g129110.1, and petunia MYBX; (ii) a MYBL1 clade of R3 repressors, including *Iochroma* MYBL1 and its eggplant ortholog, *Smel*010g336390.1, *A. thaliana* MYBL2, and (iii) a clade including R2R3 MYB repressors belonging to subgroup 4, composed by petunia MYB27, *A. thaliana* MYB4 and its eggplant ortholog *Smel*001g115870.1.

Figure 1.4: ***Phylogenetic tree of MYB transcription factors in S. melongena genome.*** *The optimal NJ tree with the sum of branch length = 45.96037176 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. This analysis involved 154 amino acid sequences, 129 from Eggplant and 25 from known anthocyanin related MYBs in other plant species. Clades containing MYB proteins involved in positive and negative anthocyanin regulation are marked with purple and blue circles, respectively.*

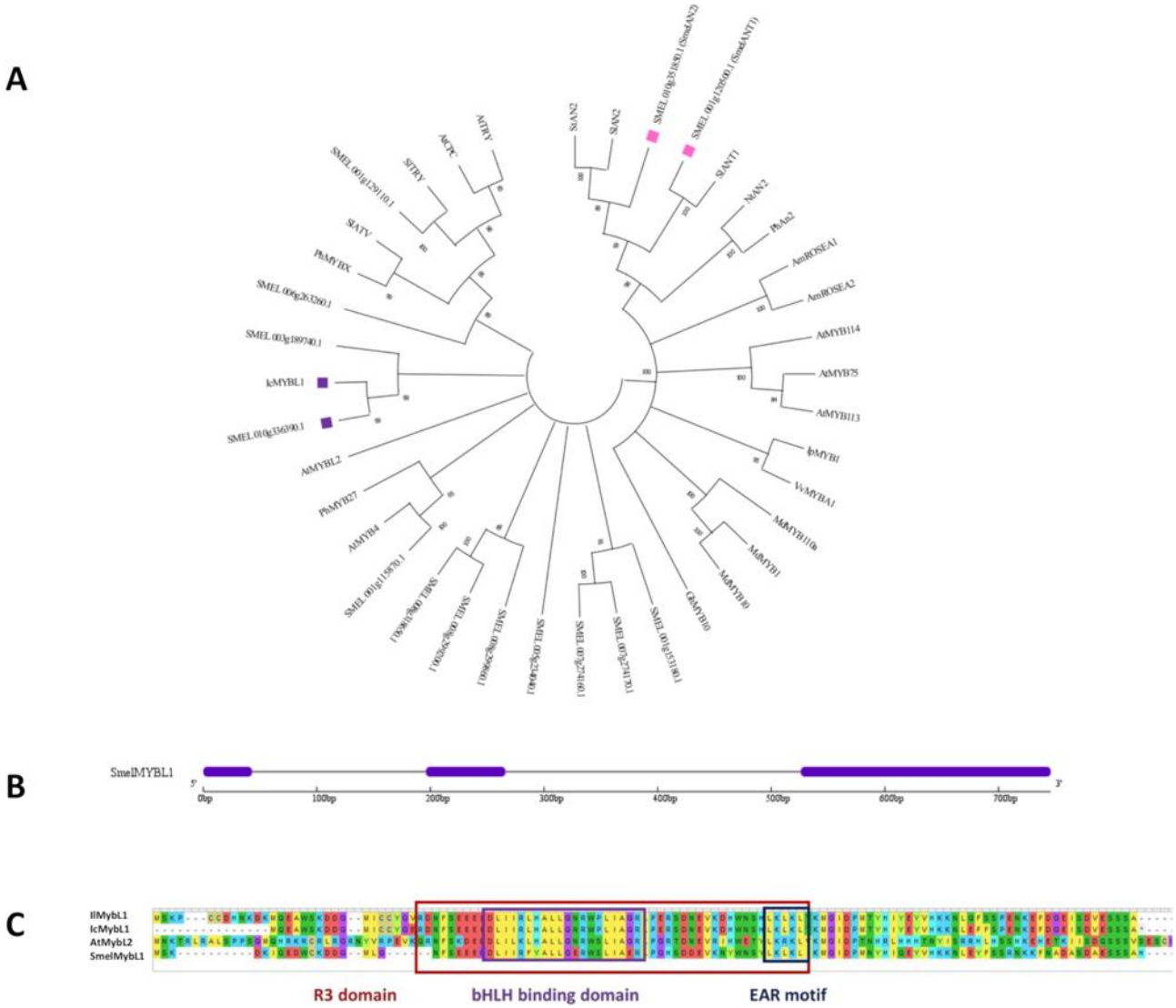

Figure 1.5: ***(A)*** *Phylogenetic tree of MYB transcription factors related to flavonoid synthesis. The optimal NJ tree with the sum of branch length = 8,61587540 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis included 39 amino acid sequences. Positive and negative candidate MYBs analysed in this work are marked with pink and purple square, respectively.* ***(B)*** *Exon/intron structure of S. melongena MYBL1 gene. The exons and introns are represented by purple boxes and black lines, respectively.* ***(C)*** *Domain structure of MYBL1 type repressors.*

According to previous works (Barchi et al., 2012; Toppino et al., 2016), in eggplant two anthocyanin related QTLs are located on chromosome 5 and 10, which respectively explain the type and intensity of anthocyanin pigmentation. We focused our attention on the candidate repressor mapping on Chr10, *SMEL*010G336391.1 (*Smel*MYBL1), whose intron-exon structure is reported in Fig 1.5B. Similarly to what observed for *Iochroma* MYBL1, *Smel*MYBL1 lost the EAR motif characteristic of the *MYB3like* genes and *petunia MYB27* (Albert et al., 2014) but acquired a new EAR motif near the end of the R3 domain (Fig 1.5C). Moreover, the *Smel*MYBL1 is characterized by the bHLH binding motif in the R3 domain and likely binds with the bHLH transcription factors acting as part of an MBW regulatory complex.

Transcriptional profiling of anthocyanin related genes

To investigate the function of *SmelANT1*, *SmelAN1*, *SmelAN2*, *SmelJAF13* in the regulation of anthocyanin synthesis, we performed their RT-qPCR expression analysis in flower at the anthesis and fruit at unripe (stage A) and commercial ripening (stage B) (Fig.1.6). The *SmelAN11* (*SMEL*003G185640.1), a WD40 encoding gene, previously identified by Barchi and colleagues (2019), along with *SmelDFR*, a structural gene of the biosynthetic pathway, were also included in the analysis. As highlighted in Fig. 1.6, *SmelDFR* resulted highly expressed at both stage A and B as well as in flower organs. As previously observed [Barchi et al., 2019], the R2R3 MYB encoding genes *ANT1* and *AN2* showed a tissue specific expression. *AN2* was poorly expressed in all the tissues except flowers,

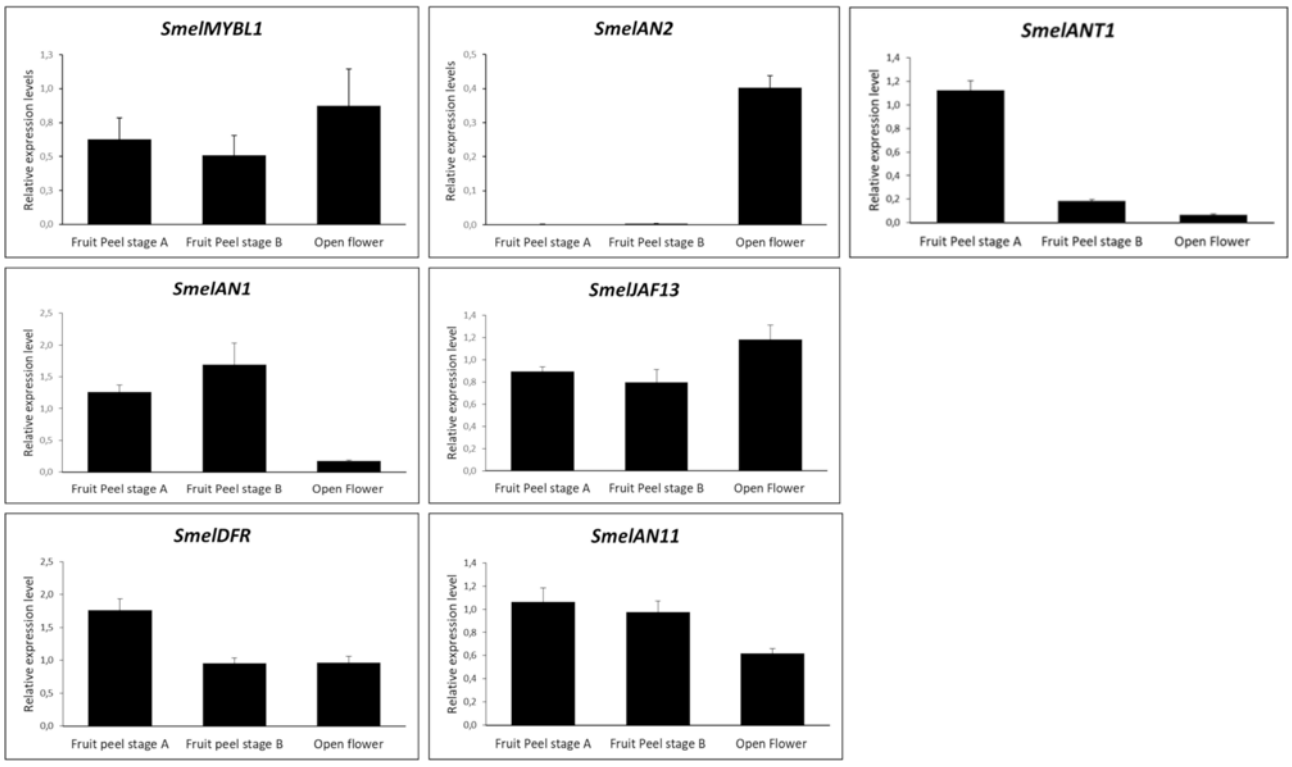

Figure 1.6: RT-qPCR based transcription profiling of eggplant MYB-L1, AN2, ANT1, AN, JAF13, DFR, AN11in two stages of fruit ripening (stage A and B) and in flower organs. Expression levels, measured by RT-qPCR, are shown as relative units using SmelGAPDH as reference gene. Data are means of three biological replicates ± SD.

while *ANT1* was highly expressed at the fruit stages A and B (Fig 1.6). These results reinforce the hypothesis of *AN2* and *ANT1* involvement in the regulation of anthocyanin synthesis in fruits and flowers respectively. Indeed, also *SlANT1* and *SlAN2* in tomato (Kiferle et al., 2015) as well as *ScAN1* and *ScAN2* in *S. commersonii* (D'Amelia et al., 2018) were found to be differently involved in anthocyanin regulation. The putative repressor encoding gene, *SmelMYBL1*, resulted to be expressed in all analyzed tissues. As previously reported (De Vetten et al., 1997; Kiferle et al., 2015), the WD40

encoding gene AN11 was found to be constitutively expressed in all the tissues analyzed. The two bHLH encoding genes, *SmelAN1* and *SmelJAF13*, were always expressed in tissues containing anthocyanins, with the former more expressed in fruits and the latter in flower organs (Fig 1.6). This suggests that multiple MYB-bHLH-WD40 complexes exert their regulatory role in different organs as highlighted in other species (Allan et al., 2018). All our RT-qPCRanalyses in eggplant tissues and organs confirm a clear correlation of *DFR* and *R2R3 MYB TF*s transcript levels with anthocyanin content, as previously reported in eggplant (Fig 1.7) (Barchi et al., 2019) as well as other species (Zhang et al., 2014; Jiang et al., 2016; Gisbert et al., 2016).

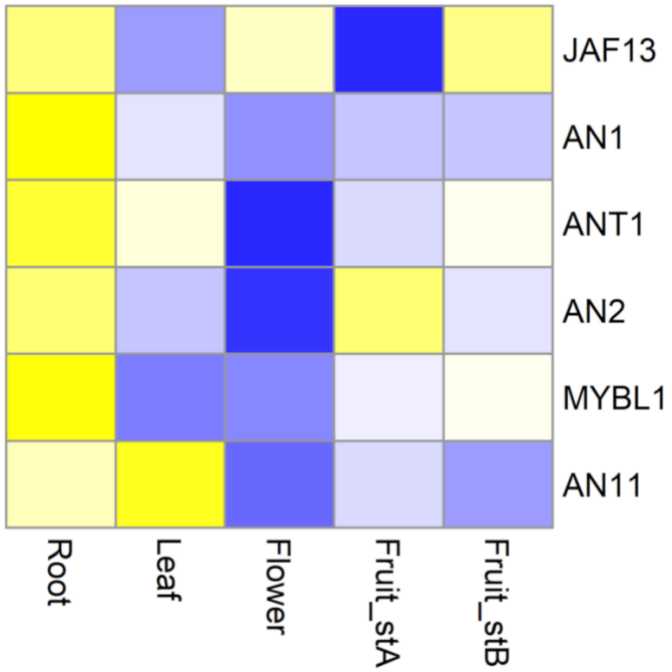

Figure 1.7: Expression value (FPKM) in 5 eggplant tissue samples: root, leaf, flower, fruits stage A (unripe), fruit stage B (commercial ripening) redrawn from data from Barchi et al., (2019). The average value is sorted by color, from yellow (low) to blue (high).

Yeast two-hybrid

Interactions between proteins belonging to MYB (*Smel*ANT1, *Smel*AN2 and *Smel*MYBL1) and bHLH (*Smel*JAF13, *Smel*AN1) families were investigated by means of a yeast two-hybrid assay (Fig 1.8). Since the fusion of anthocyanin MYB regulators with the GAL4 binding domain led to the auto activation of reported genes, these were associated to GAL4 activation domain. Thus, the coding sequence of *Smel*JAF13 and *Smel*AN1 were inserted in the bait vector, while *Smel*ANT1, *Smel*AN2 and *Smel*MYBL1 in the prey vector. Yeast cells co-transformed with *Smel*ANT1, *Smel*AN2 and

*Smel*MYBL1 in combination with *Smel*JAF13 or *Smel*AN1 grew on selective medium lacking leucine, tryptophan and uracil (Fig 1.8B), demonstrating the ability of different MYB proteins to form a complex with bHLH partners. Negative controls, consisting of yeast cells co-transformed with prey plasmids containing MYB proteins and empty bait plasmid, as well as the opposite combination (*i.e.* bait plasmids containing bHLH proteins and empty pray plasmid), did not grow on selective medium, indicating the lack of interaction. Our findings were further supported by the formation of blue colonies through beta-galactosidase assay (Fig 1.8C). These results are in accordance with those reported by D'Amelia et al. (2014), which highlighted the ability of *StAN1* (ortholog of *Smel*ANT1) to interact with StbHLH1 and StJAF13. Besides, the protein interaction assay carried out in tomato protoplasts demonstrated that SlMYB-ATV could actually bind both the endogenous bHLH factors SlAN1 and SlJAF13 (Colanero et al., 2018), in analogy with *Smel*MYBL1.

Figure 1.8 ***Y2H assay.*** *ANT1, AN2 and MYBL1 were cloned in the prey plasmid pDEST22 and transformed with the bait plasmid pDEST32 (containing JAF13 and AN1). pDEST22 and pDEST32 were used as a negative control. Yeast cells were grown for three days on (A) synthetic complete medium lacking tryptophan and leucine (-W/-L), (B) on selective medium lacking tryptophan, leucine and uracil (-W/-L/-U) and (C) on Whatman 541 filter papers, saturated 2% X-gal solution.*

Bimolecular Fluorescent Complementation (BiFC)

To confirm the interaction between AN1 and MYBs proteins, we carried out a Bimolecular Fluorescent Complementation experiment using *A. thaliana* mesophyll protoplasts as model system. The N-terminal domain of the Yellow fluorescent protein (nYFP) was fused in frame with the *Smel*AN1 CDS missing of the stop codon, while the CDSs of the three investigated MYBs, after removal of the stop codons, where fused upstream the C-terminal domain of the YFP (cYFP). Freshly isolated *Arabidopsis* protoplasts were then transformed with combinations of plasmids carrying the expression cassette for the fusion proteins and subjected to con- focal microscopy the following day. A fluorescent signal, indicating an interaction between the investigated proteins, was reported for all the combinations tested (Fig 1.9), thus confirming the ability of *Smel*AN1 to interact with *Smel*AN2,

*Smel*ANT1 and *Smel*MYBL1. Otherwise, no signal was detected in the control transformations (Fig 1.9). By means of DAPI staining, we also proved that all complexes localized into the nuclei.

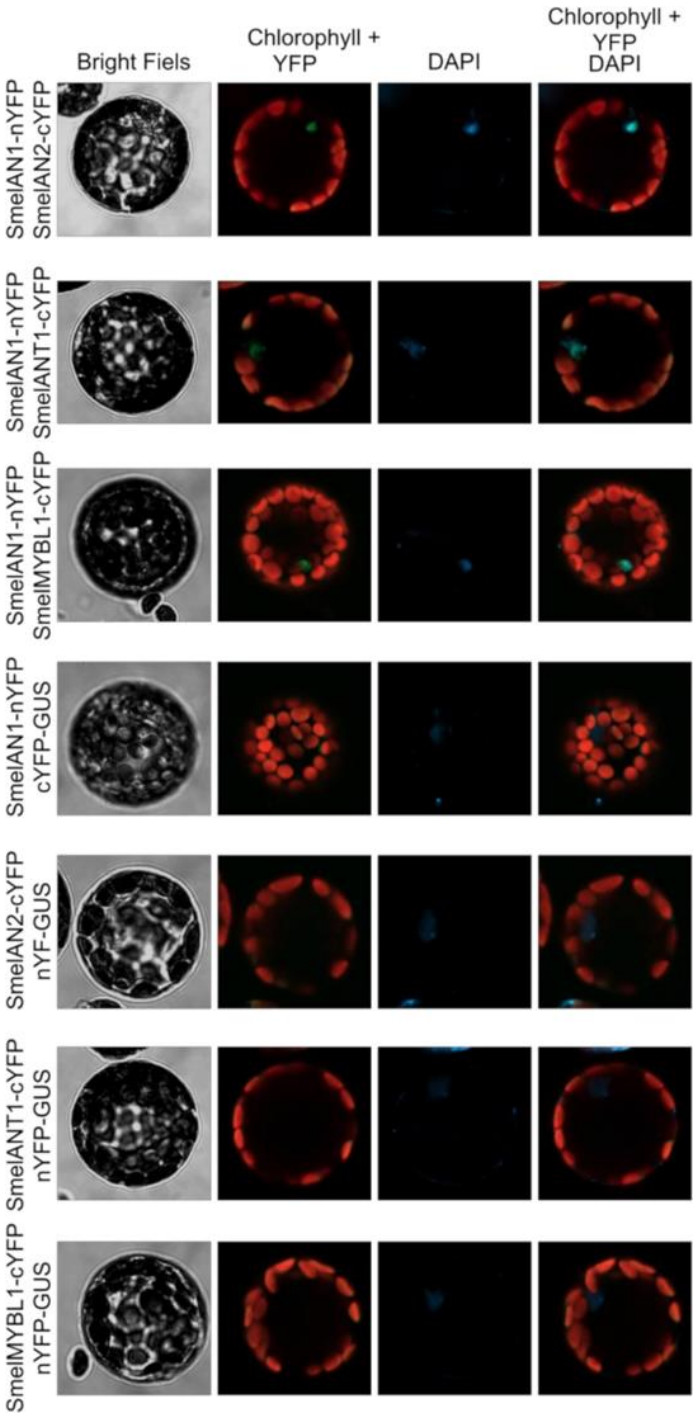

Figure 1.9 ***Bimolecular fluorescent complementation assay.*** *SmelAN1-nYFP fusion proteins was co-expressed transiently with SmelAN2-cYFP, SmelMYBL1-cYFP or SmelANT1-cYFP fusion proteins in freshly isolated Arabidopsis mesophyll protoplasts. GUS protein fused to both nYFP or cYFP was used as negative control. The cellular localization of interactions was investigated through DAPI staining.*

Transient heterologous expression in *Nicotiana benthamiana*

To verify the effect of ectopic expression of the three *Smel*MYBs and two *Smel*bHLHs *in planta*, we carried out a *N. benthamiana* leaf transient expression assay (Fig 1.10). *Agrobacteria* transformed with a pEAQ expression vector containing *SmelANT1*, *SmelAN2*, *SmelMYBL1*, *SmelJAF13*, *SmelAN1* were infiltrated individually or in combination (*MYBL1* with *ANT1* or *AN2*) in *N. benthamiana* leaves. Plants agro-infiltrated with the empty vector were used as negative controls. Four days after infiltration, an anthocyanin-pigmented phenotype was clearly visibly in pEAQ_ANT1 and pEAQ_AN2 agro-infiltrated leaves, while no anthocyanin accumulation was detected upon expression of MYBL1 as well as of JAF13 and AN1 (Fig 1.10). Interestingly co-expression of MYBL1 together with ANT1 and AN2 prevented anthocyanin accumulation. HPLC analyses on the transformed leaves were also carried out to provide additional evidence of anthocyanin accumulation (Fig 1.12).

Figure 1.10 ***The effects of over-expression of ANT1, AN2 and MYBL1 in Nicotiana benthamiana.*** *Leaves of N. benthamiana after agroinfiltration with ANT1, AN2 and MYBL1 and a combination of MYBL1 with ANT1 or AN2. Anthocyanin accumulation is indicated by dotted white circles.*

Indeed *N. benthamiana* leaves transiently expressing *Smel*ANT1 and *Smel*AN2 were found to accumulate 156.14 and 20.92 µg/g fresh weight (FW) respectively of delphinidin 3-*O*-rutinosid, which was not detectable in leaves agro-infiltared with the empty vector (Fig 1.11). In line with the visual observation, no delphinidin 3-*O*-rutinosid was detected in samples agroinfiltrated with MYBL1, JAF13 and AN1 as well after co-infiltration of MYBL1 together with *Smel*ANT1 and *Smel*AN2. Our results thus support previously transient expression in tobacco (Docimo et al., 2016) and stable expression in eggplant (Zhang et al., 2016; Kiferle et al., 2015), in which anthocyanin accumulation was verified for *Smel*ANT1. Co-expression of MYBL1 together with ANT1 prevented completely the anthocyanin accumulation, acting as a negative regulator of the biosynthetic pathway.

Analogous results were found in *N. tabacum* after the expression of *I. loxense* MYBL1, resulting in a nearly complete loss of floral anthocyanins in (Gates et al., 2018). On the other side, the co-expression of ANT1 together with *Smel*MYB44/*Smel*MYB86, proposed as negative regulators of the anthocyanin pathway, led to a decrease but not to a complete stoppage of anthocyanin production in eggplant (Li et al., 2017).

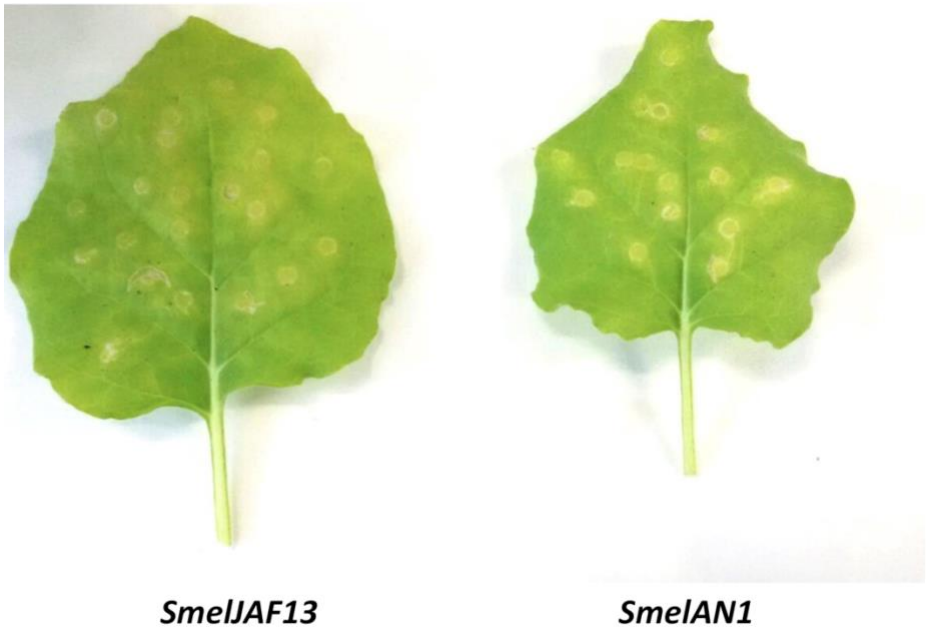

Figure 1.11: ***The effects of over-expression of JAF13 and AN1 in Nicotiana benthamiana.*** *Leaves of N. benthamiana after agroinfiltration with JAF13 and AN1.*

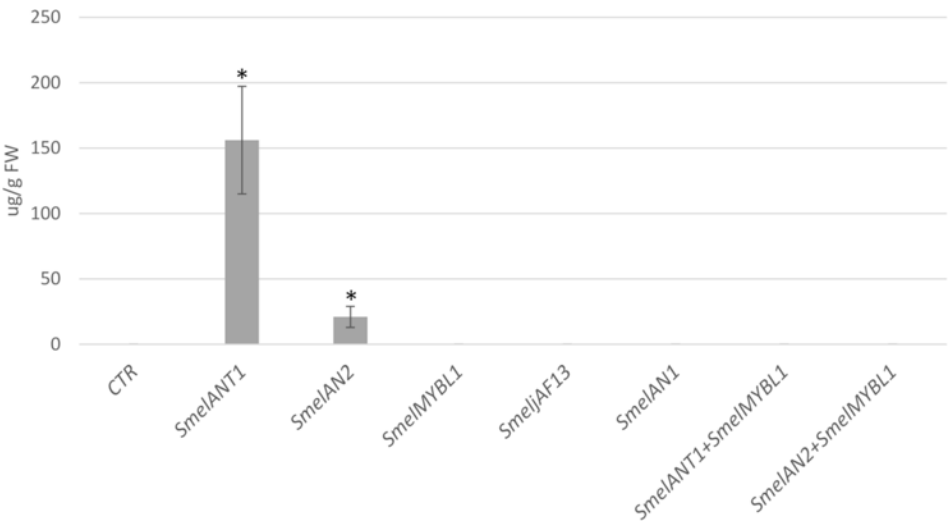

Figure 1.12: ***The effects of over-expression of ANT1, AN2 and MYBL1 in Nicotiana benthamiana.*** *Concentration of delphinidin 3-O-rutinoside in tissue extracts of N. benthamiana control (CTR) and transiently transformed leaves (ANT1, AN2, AN1, JAF13, MYBL1 and a combination of MYBL1 with ANT1 or AN2). Error bars represent SD (n = 3). Asterisk indicates significance based on Tukey's test (P□0.05).*

MYB repressors with the core MBW activation complex (Colanero et al., 2018; Cao et al., 2017; Chen et al., 2019). The replacement of one of the R2R3 MYB partners in the MBW complex with an R3 MYB may transform the complex from an activator to a repressor of anthocyanin gene transcription. We identified in eggplant the candidate TFs in the anthocyanin related MBW activation complexes. These include two MYB TFs (*Smel*ANT1 and *Smel*AN2), two bHLH TFs (*Smel*-JAF13 and *Smel*AN1) and one WDR (*Smel*AN11). *Smel*MYBL1, which belongs to R3 MYB, might represent a new component of the eggplant MBW complex. The latter appears to act as inhibitor of MBW complex by competing with MYB activators (*Smel*ANT1 and *Smel*AN2) for binding to *Smel*JAF13 and *Smel*AN1, thus hindering the chances to form new MBW complexes. In recent years the CRISPR/Cas9 system has emerged as a powerful technology for genome editing and is now widely used to explore gene function. Thanks to the ongoing development of this technology in eggplant (Moglia et al., 2019), our future goal will be to deepen the functional characterization of the isolated genes and validate MBW activation complex in the species. The anthocyanin accumulation in eggplant berries is determined by the balance between biosynthesis and degradation, thus our increase in understanding the genetic mechanisms regulating both processes may open the way for future genetic engineering approaches aimed increase the content of fruit anthocyanins through increasing their production but also through reducing their degradation.

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Chapter 2: A loss of function mutation in acyltransferase *SmAAT* causes a major difference in eggplant anthocyanin peel composition

Introduction

Anthocyanin are a class of flavonoids playing important roles in promoting pollination, seed dispersal and in protecting plants against damage from UV radiation, freezing or drought stresses, and microbial agents (Zhang et al., 2016). They are responsible for the blue, purple and red coloration of many plant tissues and are stabilized water-soluble pigments derived from anthocyanidins, principally from pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Kahkonen and Heinonen 2003). Anthocyanidins produced by the plants are modified into anthocyanins by species-specific addition of glycosyl moieties, often initiated by a O-glycosylation at the R3 position, and by further modifications, like aromatic acylation (Tanaka et al. 2008). Anthocyanins have long been considered valuable secondary metabolites for their potential as natural food coloring agents, and as they have been reported to have a beneficial effect on human health due to their strong antioxidant capacity (Cao., 1996; Lila., 2004, Mateus et al. 2008, Bendokas et al. 2020). Anthocyanins are synthetized in plants through a branching of phenylpropanoid pathway, which uses phenylalanine as precursor; this network is a highly conserved among species and one of the most studied pathways in eudicots (Albert et al., 2014; Liu et al., 2018). In dicotyledonous species the structural genes encoding enzymes involved in the biosynthetic pathway are commonly divided into two clusters: the early biosynthetic genes (EBGs) and the late biosynthetic genes (LBGs). In the first part of the pathway, through the action of the EBGs the metabolic precursors phenylalanine and 4-cumaroil-CoA are processed to produce naringenin (Holton et al. 1995). Naringenin is then converted to dihydrokaempferol, which in turn originates dihydroflavonols, the last compounds synthesized through the action of the EBGs (Pelletier et al., 1997). The enzymatic steps catalyzed by LBGs begin with the action of dihydroflavonol 4-reductase (DFR) which converts dihydroflavonols to leucoanthocyanidins, then anthocyanin synthase (ANS) originates anthocyanidins which are converted in the final form of anthocyanins.

Solanum melongena L., usually known as common eggplant, belongs to the *Solanaceae* family and is an important crop cultivated worldwide. The eggplant berry has been recognized as an important source of polyphenolic compounds mainly represented by chlorogenic acid in the flesh and anthocyanins in the peel (Mennella et al., 2012). Due to a growing interest in the nutraceutical use of vegetables rich in secondary metabolites, availability of nutrients has been included as objectives of the eggplant breeding programs (Prohens et al., 2007, 2013; Gajewski et al., 2009; Plazas et al., 2013;

Zhang et al., 2014; Docimo et al., 2016; Lo Scalzo et al., 2016). Eggplant produces the anthocyanidin delphinidin, which in different accessions is converted into the more stable anthocyanin forms delfinidine-3-rutinoside (D3R) or delphinidin-3-(p-coumaroylrutinoside)-5-glucoside (nasunin). This last step of decoration is brought by the action of an acyltransferase (AAT) that uses ρ-coumaroyl CoA as donor to acylate the rutinose residue, and of a glucosyltransferase (5GT) adding a glucose moiety in 5 position (Tigchelaar et al. 1968, Kroon *et al.* 1994; Fujiwara *et al.* 1997; Yonekura-Sakakibara *et al*. 2000; Ichinayagi *et al*. 2005; Azuma *et al.* 2008). In petunia, the 5-*O*-glucosylation does not take place unless the anthocyanin is acylated first (Jonsson et al 1984; Yamazaki et al., 2002) and it is extremely likely the same is true for eggplant. The stabilized anthocyanins are then transported to the vacuoles where they are stored in a pathway which involves the action of glutathione-s-transferase, and ABC and MATE transporters localized at the tonoplast (Mueller et al., 2000, Zhao and Dixon, 2009), or via a route which involves membrane-bound bodies (Kallam et al., 2017). As a result of the presence of these two anthocyanins, the skin color of eggplants can be either black/dark purple or dark/light lilac which generally reflect the presence, respectively, of D3R or nasunin (Tigchelaar et al., 1968, Toppino et al., 2016). The eggplant cultivars with intense anthocyanin colored fruit skin are among the most cultivated worldwide with the black ones particularly important for the Mediterranean and North America market (Munoz Falcon et al, 2009). Azuma et al. (2008) showed how HPLC profiles dominated by nasunin are typical of 'type 1' (Japanese type) eggplants while the 'type 2' accessions are characterized by the presence D3R, and how nasunin exerts a much stronger antioxidant activity compared to D3R in DPPH and Linoleic acid radical-scavenging assays. More recently, Braga et al. (2016) confirmed the higher scavenging capacity of nasunin in four assays (superoxide anion, hydroxyl radical, LACL, and Fremy's salt).

In eggplant anthocyanin formation was long thought to be a complex trait, involving several loci with assumed epistatic interactions and/or pleiotropic effects (Tatebe et al., 1939, Tigchelaar et al. 1968). More recent studies elucidated the strong dependence of the fruit peel coloration on tissue developmental stage and environment, (Mennella et al, 2012; Stommel et al., 2015; Barchi et al., 2019). Regulatory complexes comprising different MYB, bHLH and WD40 proteins are crucial in the regulation of LBGs (Petroni and Tonelli, 2011) with SmMYB1 (Zhang et al., 2014, Docimo et al., 2016) and SmMYBL1 repressor (Moglia et al., 2020) shown to be responsible for the induction/repression of the anthocyanin pigmentation. QTL-related studies employing both bi-parental mapping populations (Barchi et al., 2012; Portis et al., 2014; Toppino et al., 2016) and GWAS approaches (Ge *et al*., 2013; Cericola *et al.,* 2014) allowed the identification of two clusters of QTLs, spanning two genomic regions on E05 and E10, involved in anthocyanin pigmentation in all eggplant tissues and organs including flowers, vegetative parts and fruit peel. The QTLs belonging

the cluster on E10 appear mainly involved and anthocyanin intensity and distribution in all the vegetative tissues as well as in color determination of the peel under the fruit calyx and may be ascribable to some QTLs previously identified by Doganlar *et al*. (2002). Conversely, the QTLs belonging to the cluster on E05 are involved both in fruit and corolla color, in the determination of anthocyanin tonality as well as in the alternative production of D3R or nasunin in the fruit peel. The first high quality eggplant genome sequence of '67/3', a F8 breeding line characterized by the presence of nasunin, was recently released and a resequencing of '305E40', a double-haploid line producing D3R, was also carried out (Barchi et al., 2019b). These resources, together with a recently developed ultra-high-density genetic linkage map (Toppino et al., 2020) and metabolomic analyses on fruit peel composition (Sulli et al., 2019), allowed to narrow the confidence intervals of QTLs associated with anthocyanin pigmentation from that indicated in the previous papers (Toppino et al., 2016, Portis et al., 2014; Barchi et al., 2012) and to identify candidate genes responsible of the traits. The major QTL associated with anthocyanin tonality is now located in a region on chromosome E05 at around 36.3 Mb (Toppino et al., 2020), close to marker 3311_PstI_L361 (E05:36.94 Mb) previously reported as the main QTL marker for both tonality and composition of anthocyanin (Toppino et al., 2016). In this region the gene SMEL_005g236240 (Ch05:36,803,460), annotated as an *Acetyl-CoA-benzylalcohol acetyltransferase* (*SmAAT*) was proposed as the most convincing candidate gene for the conversion of D3R into nasunin (Toppino et al., 2020). Here, the genetic determinant putatively involved in the differential accumulation of the anthocyanin D3R or nasunin has been deeply investigated in 67/3 and 305E40, two eggplant accessions belonging to the "Type 1 -Japanese" and "Type 2", respectively. A loss-of-function indel variant of the *SmAAT* gene sequence in 305E40 was identified as possibly causative of the retention of D3R in this line. Genetic and biochemical characterization of segregating populations obtained from the cross with the sequenced nasunin-containing line 67/3 evidenced that the identified allelic variants correlate with the Nas-D3R phenotype, and this was further confirmed in a wider collection of eggplant accessions belonging to both typologies and characterized by different peel color. Nasunin accumulation restoration was detected in D3R-containing lines (305E40 and DR2) transformed to express the coding sequence of the active allele of *SmAAT* gene from 67/3, thus demonstrating the pivotal role of SmAAT in eggplant anthocyanin decoration. Furthermore, RT-qPCR analyses were conducted with the aim of identifying potential changes in the expression of genes putatively involved in the last steps of the anthocyanin decoration in the eggplant fruit peel. Alongside *SmAAT*, the expression of the putative S*m5GT1* responsible for nasunin production was assayed. Additionally, the *S. melongen*a orthologues of two genes previously described to be involved in the last step of anthocyanin decoration in other plants were included in this analysis: *Anthocyanin-methyltransferases (AMT*) identified petunia

(Provenzano et al., 2014) and *Flavonoid-methyltransferase (FMT)* reported in studies on *A. thaliana* (Poulton et al., 1977; Saito et al., 2013).

Materials and methods

Plant materials and growth conditions

Several plants of *S. melongena* lines '67/3', '305E40' and 'DR2' were grown in pots (30 cm diameter) in glasshouse at CREA-GB (Montanaso Lombardo - Italy), under standard conditions, in 2017-2019. '305E40' is a double haploid line derived from an interspecific somatic hybrid *Solanum aethiopicum* gr. *gilo* (+) *S. melongena* cv. Dourga (Rizza et al. 2002), which was repeatedly backcrossed with the recurrent lines DR2 and Tal1/1, prior to selfing and final anther culture. It produces pink flowers and long, highly pigmented dark purple fruits characterized by the presence of the anthocyanin D3R. "67/3" is an F_8 selection from the intra-specific cross cv. 'Purpura' x cv. 'CIN2'; it displays higher anthocyanin pigmentation than '305E40' in leaves and stems, and produces violet flowers and round, dark lilac colored fruits with white peel color under and next to calyx. The fruits are characterized by the presence of the anthocyanin nasunin in the peel.

'DR2' is a breeding line utilized as recurrent parent in some steps the breeding program which yielded the line 305E40 and is routinely utilized in our lab for genetic transformation as it usually yields a good transformation frequency. It produces pink flowers and long, dark purple fruits characterized by the presence of the anthocyanin D3R.

Three plants of each of the 69 accessions from the eggplant germplasm collection available at CREA (detailed in table 2.2), selected as displaying a wide range of different peel colors, were grown in pots (30 cm diameter) in glasshouse at CREA-GB (Montanaso Lombardo - Italy), under standard conditions from March to September 2018. The fruit color of all accessions was evaluated using at least two representative fruits per plant, collected at the commercial stage, according to the following scale: 0=dark green; 1=green; 2= milk white; 3=yellow; 4=fire red; 5=scarlet red; 6=lilac; 7: dark lilac; 8=purple; 9=dark purple; 10= black. Leaves were collected from all accessions bred in greenhouse, immediately freezed in liquid nitrogen and stored at -80°C. Samplings for biochemical analyses were carried out at two fruit ripening stages, unripe (stage A), approximately 21 DAF (days after flowering), and commercial ripening (stage B), approximately 38 DAF, according to Mennella et al. (2012).

Molecular phylogenetic analysis by neighbor joining method

Peptide sequences from different species used for phylogenetic analysis were obtained through BLASTp by using the SmAAT protein sequence as bait in the NCBI database

(https://www.ncbi.nlm.nih.gov) to obtain *S. lycopersicum*, *S. tuberosum* and *Capsicum annuum* sequences, the Sol genomics database *(https://solgenomics.net)* for *Petunia axillaris* and the eggplant genome database (http://www.eggplantgenome.org) for *S. melongena* using $1e^{-60}$ as E-value cut-off. The *Vitis vinifera* sequence *XP_010648156* (identical to *Vv3AT,* Rinaldo et al. 2015) *w*as also obtained from NCBI and used as an outgroup. Phylogenetic relationships were estimated in MEGAX (Kumar et al., 2018). Peptide sequences were aligned by MUSCLE with default settings. Evolutionary relationships among TOE-type members were inferred by using the neighbor joining method based on the JTT matrix-based model. The rate variation model allowed for some sites to be evolutionarily invariable and a discrete Gamma distribution was used to model evolutionary rate differences among sites. The reliability of the phylogenetic tree was estimated by setting 500 bootstrap replicates.

DNA extraction and HRM analysis

DNA samples were extracted from young leaves, using the GenEluteTM Plant Genomic DNA Miniprep kit (Sigma, St. Louis, MO), following the manufacturer's protocol. Genotyping was carried out via the High Resolution Melting (HRM) technique [Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ (2003) High-Resolution Genotyping by Amplicon Melting Analysis Using LCGreen. Clin Chem 49: 853-860] utilizing the EvaGreen supermix kit (Biorad) according to the manufacturer's protocol and ran using a Rotor-Gene 6000 (Corbett Research, Mortlake, NSW, Australia) PCR machine utilizing the HRM™ with pre-Amplification program. Primers for HRM are detailed in table 2.1. Both melting curve (range 50-80°C with increment of 1°C per cycle) HRM (55-70°C, increment of 0.2°C per cycle) function were utilized for analysis of genotyping data. HRM haplotype was scored as NASU(1), D3R (0), H (2), multiple (++),or different peak (3)

Cloning of the *Smel_AAT* gene

The Smel_AAT (Smel005g236240.1.01) sequence was amplified by PCR starting from total cDNA eggplant peel both from 67/3 and 305E40 accessions using the "Phusion High-Fidelity DNA Polymerase" (Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's protocol. Primers for amplification of the complete sequence in both parental lines and cloning are detailed in Table 2.1. The amplified genes were sequenced for confirmation. Multiple Sequence Alignments were performed using Multiple Sequence Alignment by CLUSTALW.

Table 2.1: List of primers used in this chapter.

Gene	Sequences	Scope

SmAAT	FW 5'- atgagccaaattacaaaacaaaacttaaatg -3'	HRM
	RV 5'- aggcacagcaaaattaggagc -3'	
SmAAT Seq	FW 5'- atgagccaaattacaaaacaaaactta -3'	Sequencig
	RV 5'- tccaatcccatcagccatca -3'	
SmAAT GW	FW 5'- caccatgagccaaattacaaaacaaaa -3'	Gateway cloning
	RV 5'- tagaaattccaaaatctttcaag -3'	
NPTII	FW 5'- tgctcctgccgagaaagtat -3'	PCR
	RV 5'- agaactcgtcaagaaggcgatag -3'	
SmAAT	FW 5'- cgtgcaaatggtccacacat -3'	RT-qPCR
	RV 5'- ttccccttccccatacgact -3'	
Sm5GT1	FW 5'- tccattcaacttcctggcct -3'	RT-qPCR
	RV 5'- aggcttccttttgcacttga -3'	
SmAMT	FW 5'- cttccgaatacagcctctcc -3'	RT-qPCR
	RV 5'- tttcacaactggggtctggg -3'	
SmFMT	FW 5'- ggaggcaaagaaaggacgga -3'	RT-qPCR
	RV 5'- agcacaacaagccttacgga -3'	
SmAAT_3'UTR	FW 5'- attgaagctagagtttgctt -3'	RT-qPCR endogenous gene
	RV 5'- aataaggtacgctcccagaa -3'	
SmAAT_35S::3'UTR	FW 5'- attgaagctagagtttgctt -3'	RT-qPCR exogenous gene
	RV 5'- gctgaacttgtggccgttta -3'	
SmGAPDH	FW 5'- ggtgccaagaaggttgtgat-3'	RT-qPCR
	RV 5'- ccaatgctagttgcacaacg-3'	
Sm18S	FW 5'- atgataactcgacggatcgc -3'	RT-qPCR
	RV 5'- aaacggctaccacatccaag -3'	

RNA extraction, cDNA synthesis and primer design

Total RNA from all the eggplant peel samples was extracted using the TRIzol RNA Isolation Reagents (Thermo Fisher Scientific) combined with the Spectrum Plant Total RNA kit (Sigma Aldrich), each sample was resuspended in a final volume of 30 ul. From the total RNA extracted, 8 ul were treated with the RQ1 RNase-Free DNase Kit (PROMEGA). The single strand cDNA was synthesized from 1 µg of RNA using the ImProm-II™ Reverse Transcription System Kit by PROMEGA. The standard procedure was changed by adding both 0.5 µg/reaction of Oligo(dT) and

0.5 µg/reaction of Random Primers to 1 µg of DNase treaty RNA and heating at 70 °C for 5 min. The RNA mix was cooled to 25 °C and 15 µl of RT Master mix (according to the manufacturer's instructions) was added, followed by heating to 42°C up to 1hrs and 75 °C for 10 min to inactivate the Reverse transcriptase. Primers for RT-analysis of *SmAAT, Sm5GT1, SmAMT e SmFMT* detailed in Table 2.1 were designed basing on the available genome sequence of 67/3 utilizing the Primer 3 software (https://bioinfo.ut.ee/primer3-0.4.0/primer3/). For expression analysis of *AAT* in the transformed plants, two additional primers were designed to distinguish the expression of the endogenous version of the gene (either *SmAAT* or *305E40_aat)* from the exogenous *p35s::SmAAT* transcript. For this use, a common forward primers was placed on the last exon of the *AAT* gene while the specific reverse primers were positioned on the 3*'UTR* region of the endogenous gene or the 3'UTR of the p*35s::SmAAT* construct, respectively.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) setup.

The RT-qPCR reactions were carried out according to the following PCR parameters: 95°C for 5 min, followed by incubation for 15s at 95°C and denaturation for 15s at 95°C, annealing for 60s at 59°C for 40 cycles, followed by elongation at 72°C for 20s. The reaction was performed using GoTaq® RT-qPCR Master Mix by PROMEGA. The reaction containing 1.0 µl of previously diluted cDNA (1:20), from 0.2 µl to 1.0 µl of primers (1 µM each), 5 µl of GoTaq® RT-qPCR Master Mix and RNase-Free water up to the final volume of 10 µl. All samples were run in three technical replicates, and no-template controls were included in all analysis. Standard curves for each primer pair were calculated across a 5-fold dilution series of pooled diluted cDNA amplified in technical triplicate. The PCR efficiency was calculated by Rotor-Gene 6000 Series Software and it was optimized to be in the range 90-100% with R2-values of 0.996. The expression levels of the target genes were determined in all samples in triplicates, and relative expression ratio was calculated using the "Delta-delta method" using *SmelGADPH* (Glyceraldehyde 3-phosphate dehydrogenase) and *Smel18S* as housekeeping genes (Barbierato et al 2017). Specificity of amplifications was assessed first by PCR for the presence of a single band and then through the melt curves analysis. The analyses were performed on three biological replicates and in technical triplicates.

Construct preparation

Full sequence of *SmelAAT* from 67/3 was amplified from total cDNA of 67/3 (primers in Table 2.1) and cloned into pENTR/D-TOPO vector (Thermo Fisher Scientific); the entry clones were recombined with destination vectors pK7WG2.0 containing the constitutive 35S promoter via Invitrogen TM Gateway TM recombination cloning technology (Thermo Fisher Scientific). The

binary vector pK7WG2 containing a NPTII selection cassette flanked by T-DNA border sequences containing the *35S::SmAAT* version of 67/3, were used to transform *Agrobacterium tumefaciens* strain GV2260.

Eggplant Transformation

The procedure for eggplant transformation of cotyledon from *in vitro* grown plantlets of '305E40' and 'DR2' genotypes (both having the *305E40_aat* gene version) was essentially as described previously (Rotino et al., 1990 and 1997). Cotyledons explants were pre-cultured for two days in a pre-culture medium (Arpaia et al., 1997). For explants infections, an overnight *Agrobacterium tumefaciens* liquid culture was centrifuged and the pellet resuspended at 0.1 OD600 in MS basal medium, 2% glucose, 200mM acetosyringone pH 5.5. All the explants were infected by dipping in bacteria suspension for 5 min, blotted dry onto sterile filter paper and placed back in the same plates. After 48h the explants were transferred to selective medium without acetosyringone and supplemented with 30mg/L kanamycin and 500mg/L cefotaxime. Calli with compact green nodules were then transferred to regeneration medium. Regenerated shoots were rooted and propagated in V3 medium (Chambonnet, 1985) without antibiotics. Plants material for transformation, calli and transformed sprouts were grown in *in vitro* conditions in a growth room chamber with 16hr of daylight at ~50 μE m^{-2} s^{-1} intensity and day/night temperature regimes on ~ 25/20 °C. Kanamycin-resistant transgenic plantlets were confirmed by PCR for the presence of the insert, using the primers NPTII fw and NPTII rev (see Table 2.1). The PCR-positive transformed plants regenerated from the "*in vitro"* culture (T0), after *ex vitro* adaptation, were potted and grown under glasshouse condition for phenotypic-genotypic evaluation and self-pollinated during the summer season of 2018; the seeds obtained (T1) were sown at the beginning of summer season 2019 in plateau of 104 holes and seedlings were sprayed with kanamycin (Sunseri et al 1990). The resistant T1 plants were checked by PCR for the presence of the transgene. At least 3 Kan^R T1 plants from each T0 event were grown until fruiting.

Anthocyanins extractions and HPLC analytical conditions

Fresh skin samples were obtained using surgical scalpel blades from fruits of T0, T1 and un-transformed controls. For each single fruit, 4 samples of 0.1 g of skin were cut, immediately frozen in liquid nitrogen, stored at -80°C and then lyophilized. The freeze-dried tissues were powdered and held at −80 °C. The anthocyanins extraction was carried out through a double methanol extraction according to Mennella et al., (2012); a first extraction was performed on 100 mg of lyophilized and powdered peel using 1.0 mL of methanol then the samples were decanted; after centrifugation and

collection of the supernatant, the pelletted tissue sample was submitted to a second round of extraction with1.0 mL of methanol. The two extracts were combined, filtered through a Whatman N° 4 filter paper and then through a 0.2 µm PTFE syringe filter. Filtered extracts were stored in capped brown glass vials at -80 °C until removed for High Performance Liquid Chromatography (HPLC) analysis. RP-HPLC analysis was performed according to Mennella et al., 2012 through a Waters E-Alliance HPLC system constituted by a 2695 separations module with quaternary pump, autosampler, and a 2996 photodiode array detector; data were acquired and analyzed with Waters Empower software on a PC. The chromatographic separations were performed at a flow rate of 0.8 mL/min and at 0.1 AUFS. Purified D3R (Polyphenols Laboratories AS, Sandnes, Norway) was used as external standard in RP-HPLC analyses. As for nasunin quantification, a partially purified standard was used according to Lo Scalzo et al. (2010). The results are expressed in Nasunin molar ratio, using the following equation:

$$\text{Nasunin molar ratio} = \frac{\text{Nasunin mM}}{\text{Nasunin mM} + \text{D3R mM}}$$

The molar ratio takes into account the difference between the molecular weight (MW) of delfinidine-3-rutinoside (MW: 647.0 g x mol^{-1}) and the delphinidin-3-(pcoumaroyl-rutinoside)-5-glucoside (MW: 919.8 g x mol^{-1}), so that in this way the number of the two molecules can be compared. Moreover, expressed in mg x100$g^{-1}$of dry weight, the samples show a certain degree of variability in the total amount of accumulated anthocyanins dependent on the peel pick-up point.

Statistical analysis

Statistical analysis was performed using the JASP software (https://jasp-stats.org/) and the graph was realized whit GraphPad Prism 9. Oneway ANOVA with Tukey's HSD post hoc test was performed. Different letters indicate significant differences at $P < 0.05$. The data are means of at least three biological replicates.

Results and Discussion

Identification of a candidate variant for nasunin or D3R accumulation

Previous works identified SMEL_005g236240 encoding the *SmAAT* as the most reliable candidate gene potentially responsible for the difference in anthocyanin tonality between the "Type 2" eggplant line 305E40, characterized by blackish purple fruits and producing D3R, and the "Type 1" line 67/3, producing violet berries characterized by the presence of nasunin (Fig. 2.1). To elucidate the phylogenetic relationship of SmAAT among eggplant acyltransferases, protein sequences available from *S. melongena* and other Solanaceae were analysed. A blast search of the SmAAT protein sequence against the '67/3' eggplant genome (Barchi et al., 2019) and the NCBI and Sol Genomics databases allowed to identify putative acyltransferase amino acid sequences from *S. melongena*, *S. lycopersicum*, *S. tuberosum*, *Capsicum annuum*, *Petunia axillaris*, which were used to obtain a molecular phylogenetic tree (Fig. 2.2).

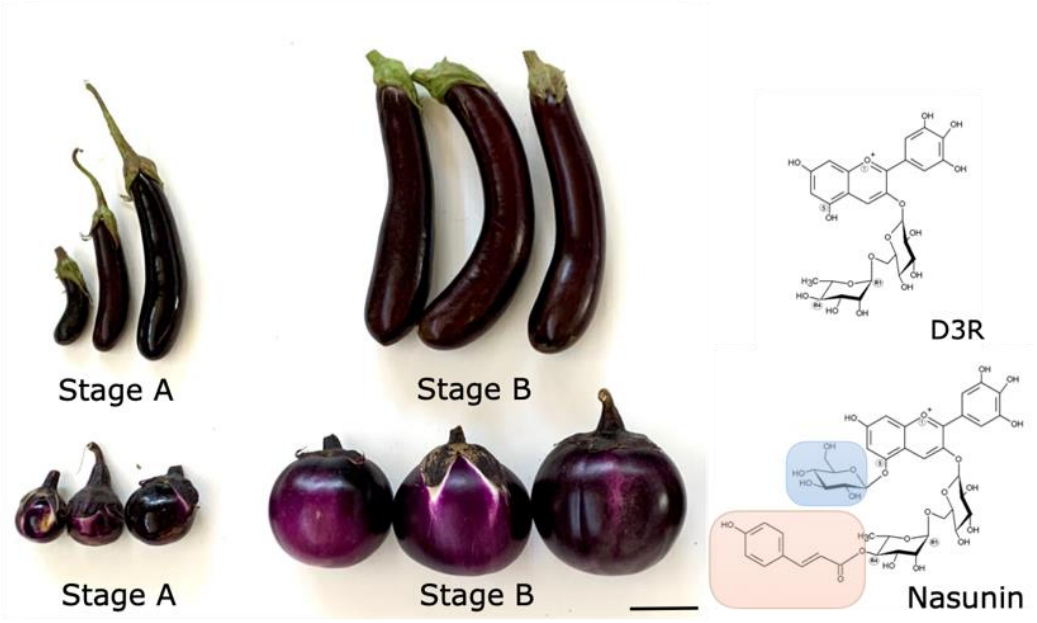

Figure 2.1 Phenotypic differences of 67/3 and 305E40 S. melongena fruits at stage B (commercial ripe, ~40 DAP). (A) Berry of 305E40 and chemical structure of delphinidin-3-rutinoside (D3R). (B) Berry of 67/3 and chemical structure of delphinidin-3-[p-coumaroylrutinoside]-5-glucoside (Nasunin). The differences between D3R and Nasunin are shaded in red (ρ-coumaroyl acylation of the rutinose residue), and in blue (5-O-glucosylation). The reference at the bottom indicates 5 cm.

Some of the sequences analyzed could be identified as corresponding to previously described acyltransferases with known function: SlAT2 (NP_001266253, Solyc01g105580), capable of acyl sucrose acetylation (Schilmiller *et al*, 2012), *SmSpmHT* (SMEL_010g340090) with spermine hydroxycinnamoyl transferase activity (Peng *et al.* 2019), and SlFdAT1 (NP_00134419,

Solyc12g088170), acting as a flavonoid-3–O-rutinoside-4‴–O-phenylacyl transferase (Tohge *et al.* 2015).

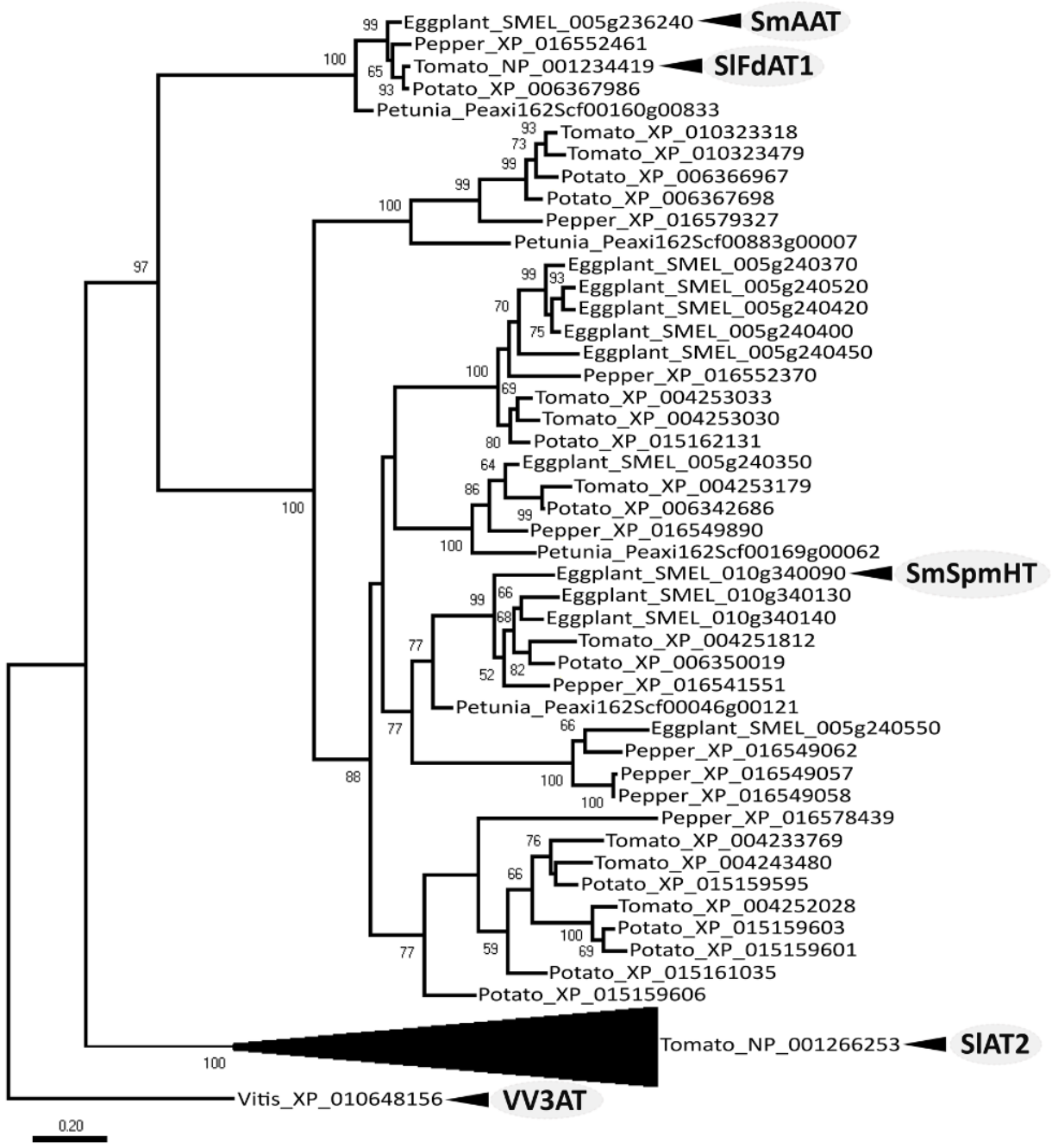

Figure 2.2 Evolutionary relationships among acyltransferase proteins in different plant species. The tree was obtained using peptide sequences from S. melongena (Eggplant), S. lycopersicum (Tomato), S. tuberosum (Potato), Capsicum annuum (Pepper), Petunia axillaris (Petunia) and Vitis vinifera (Vitis). SmAAT and other acyltransferases with known function from the literature (SlAT2, SmSpmHT, SlFdAT1) were indicated next to the corresponding sequences. A previously described V. vinifera sequence (corresponding to Vv3AT) was included as the outgroup. A subtree comprising the 32 most distantly related sequences to SmAAT was compressed for clarity. Only bootstrap values >50 are shown.

Phylogenetic analysis placed SmAAT in a small clade comprising a single sequence derived from each of the species taken into consideration, one of which being SlFdAT1 (Solyc12g088170), which can use *p*–coumaroyl CoA to acylate D3R (Tohge et al., 2015). The existence of this small clade

separated from other anthocyanin phenylacyltransferases within the Solanaceae, and the proximity of SmAAT and SlFdAT1 is a strong evidence for predicting a common role of SmAAT in the conversion of D3R into nasunin in eggplant. A deep *in silico* investigation of the genetic differences between the 67/3 reference genome and 305E40 sequences already available in the *S. melongena* genome paper (Barchi et al., 2019) allowed to highlight the presence, among more than 31'000 polymorphisms (detected both at homozygous/heterozygous states with silent, missense and nonsense impact on the predicted protein functionality), of a homozygous G nucleotide frame shift deletion in the 305E40 *SmAAT* sequence, with a predicted severe negative impact on the protein functionality. Therefore, cDNA sequence comparison between the two *SmAAT* variants isolated from peel of the two contrasting accessions '67/3' (named *SmAAT*) and '305E40' (named *305E40_aat*) confirmed the single G deletion in the latter in position 49/1359, causative of the formation of a stop codon after just 18 amino acids in the predicted translated sequence (Fig 2.3). The resulting predicted peptide lacks both CoA-dependent acyltransferase domains (CATH Domain 2bghA01, *www.cathdb.info*) and, therefore, represents a *null* allele for the SmAAT function and a putative variant for the different type of anthocyanin produced in 67/3 and 305E40.

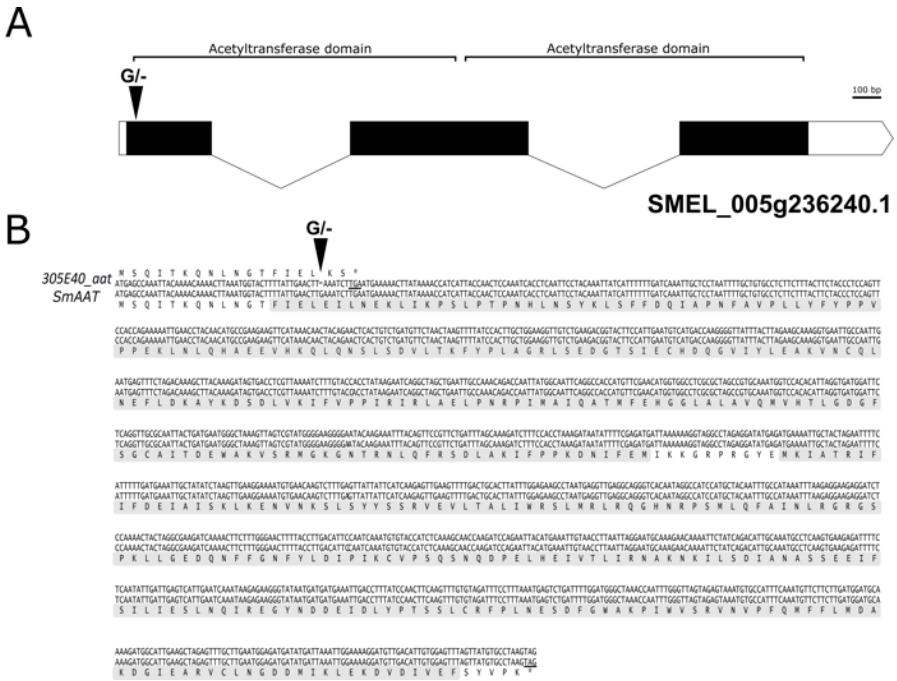

Figure 2.3 Sequence variants of the S. melongena 305_aat and SmAAT genes. (A) Gene structure of SMEL_005g236240.1 (SmAAT). Black solid boxes indicate the CDS exons, white boxes UTR regions. Gene portions encoding for the acetyltransferase domains are shown. The arrowhead indicates the position of the identified single base G deletion. (B) Comparison of the CDS and translated amino acidic sequences of SmAAT and the 305_aat allele. Acetyltransferase domains re shadowed.

An AAT-HRM marker for the variant on *SmAAT* indel correlates with the Nas-D3R phenotype

To develop a genotyping tool on the AAT allelic variants putatively responsible of the two different anthocyanin types, primers for HRM analysis were designed to amplify a region of around 150 bp around the G indel within the *SmAAT* genomic sequence. Melt analysis revealed the presence of a distinguishable single peak differentiating the 67/3_AAT (NASU) and 305E40_aat (D3R) allelic variants, and of a double peak in the F1 hybrid (H) (Figure 2.4).

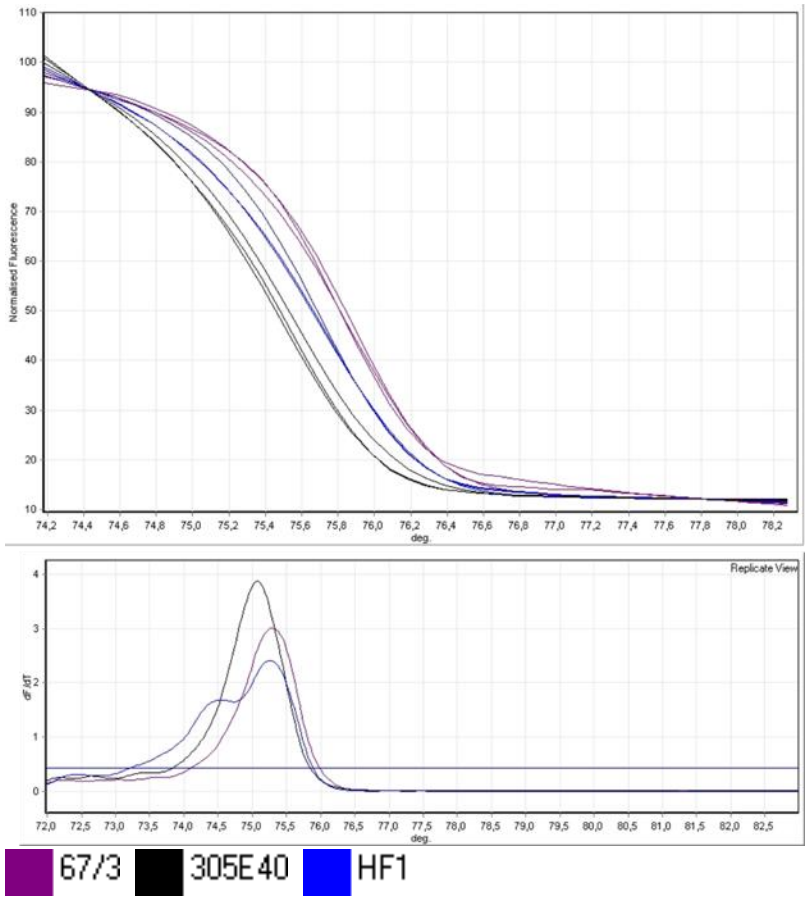

Figure 2.4: Output of Rotor-Gene 6000 Series Software 1.7 for the Melting curve of the AAT_HRM Marker. Melt analysis revealed the presence of a distinguishable single peak differentiating the SmAAT of in line 67/3 (NASU), 305E40_aat (D3R) allelic variants, and of a double peak in the F1 hybrid (H).

Validation of the ability of the AAT-HRM marker to predict the presence of nasunin or D3R was performed by genotyping 84 F2 individuals from the cross 305E40x67/3, for which the visual scoring of peel colour phenotype phenotypical and biochemical NASU/D3R composition data were already available (Toppino et al., 2016). The AAT-HRM marker showed a perfect correlation with the F2 biochemical phenotypes and a 3:1 segregation ratio (chi-square =5.2143) as confirmed by the allelic dominance of *SmelAAT* over *305E40_aat*, associated with the presence of NAS in heterozygous F1 hybrids (Toppino et al., 2016). This analysis also allowed to test the ability of the visual scoring of

peel colour phenotype commonly used in eggplant selection, revealing that 4/84 F2 individuals were phenotypically mis-annotated in this population, due to fruits characterized by a particularly dark skin colour and therefore difficult to distinguish between blackish lilac and blackish purple. Furthermore, the genic marker proved to be strongly associated (R=0,87) with the phenotype of additional 61 accessions (Table 2.2). Running an additional HPLC analysis on the accession for which the blackish peel colour made particularly difficult to score them as lilac or purple enabled to correct their phenotypic data according to the biochemical results, and the correlation between marker and phenotype further increased to R = 0.91. These results show that the *305E40_aat* allele is widespread in the tested germplasm.

Table 2.2: *Validation of HRM_AAT marker in different anthocyanic coloured CREA accessions. For each accession: Common name, accession code, HRM haplotype (SmelAAT = A, 305E40_aat = a, AAT unknown variant=AATv), peel colour (Lilac=L, Purple=P), HPLC phenotype (nasunin = NAS, delphinidin-3-rhutinoside = D3R). Accessions with no correspondence between genotype and phenotype (either visual and/or HPLC) are marked with (*).*

Name	Code	HRM Haplotype	Peel visual colour phenotype	HPLC phenotype
DADALI	AM 001	AA	L	-
CIMA VIOLA	AM 004	aa	P	-
1F5(9)	AM 010	aa	P	-
CCR3	AM 013	aa	P	-
VIOLETTA SAIS	AM 014	AA	L	-
LUGA 063	AM 015	aa	P	-
PROSPEROSA	AM 016	AA	L	-
LUNGA VIOLETTA	AM 018	AA	P*	NAS
TAL1/1	AM 021	aa	P	-
ANGIO	AM 022	AA	L	-
DR2	AM 026	aa	P	-
FANT E13D	AM 029	AA	P*	D3R*
SNL 600	AM 034	AA	L	-
CIN 01/24-6	AM 035	AA	P*	D3R*
VIOLA CIN	AM 036	AA	L	-
V. TOSCANA	AM 037	AA	L	-
44074	AM 042	AA	L	-
55-08	AM 045	AA	L	-
16-09	AM 046	AA	L	-
P621-08 *(74-4)*	AM 047	AA	L	-
P328	AM 053	AATv	P	D3R*
S1052-08	AM 056	AATv	P	D3R*
LS 3805 MINDEN	AM 086	AA	L	-
LS611	AM 103	aa	P	-
NAGA UNGU	AM 106	AA	L	-
N286	AM 107	aa	P	-
N24	AM 110	aa	P	-

N243	AM 111	aa	P	-
N286	AM 112	aa	P	-
N321-14	AM 113	Aa	L	NAS
PI17	AM 124	aa	P	-
LUNGA MARINA	AM 139	aa	P	-
BUIA	AM 156	aa	P	-
ANK2	AM 158	aa	P	-
ANGIO3	AM 167	AA	L	-
SM 19/14	AM 170	aa	P	-
PALERMITANA	AM 171	AA	L	-
JM	AM 174	AA	L	-
THAI TH472	AM 179	aa	P	-
LISTADA	AM 180	aa	P	-
THAI TH449	AM 190	aa	L*	D3R
THAI TH4760	AM 199	aa	P	--
TOPAK	AM 217	aa	P	--
PI169648	AM 221	aa	P	-
TOPATAN	AM 222	aa	P	-
PI171859	AM 232	aa	P	-
L129	AM 268	AA	L	-
DRS4	AM 274	aa	P	-
CAAS 6	AM 280	AA	L	-
CAAS 16	AM 290	AA	L	-
CAAS 17	AM 291	AA	P*	NAS
LONGO	AM 300	aa	P	-
TALINDO purple	AM 302	aa	P	-
BANGLADESH	AM 318	aa	P	-
USTICA	AM 322	AA	L	-
INDIA1	AM 360	AA	L	-
L316	AM 371	aa	P	-
LP742	AM 378	AA	L	-
L. VIOLA MEDIA	-	aa	P	-
ANOMINORI	-	AA	L	-
VIOLA OVALE	-	AA	L	-

To further validate the AAT-HRM marker on different genetic backgrounds, we genotyped further 64 accessions belonging to the germplasm collection available at CREA and chosen for their different peel pigmentation (some of which already characterized for anthocyanin composition in Mennella et al., 2012), and two RIL progenies from the cross between 305E40 x 67/3 still segregating for peel (lilac/purple) color. Comparison between genotypic data and phenotypical evaluation confirmed a high correlation (R=0.75, Table 2.2) between HRM haplotype and peel color confirming the suitability of the marker for selection of genotypes with different anthocyanin pigmentation in breeding programs. The marker resulted less effective only for discriminating accessions

characterized total black peel: an additional biochemical characterization of the peel of these accessions is planned to uncover the anthocyanin composition of the berries as they are visually undistinguishable, with the purpose to better evaluate the reliability of the AAT-HRM marker.

Complementation of *SmAAT* induces nasunin production in "Type 2" genotypes homozygous for the *305E40_aat* allele

To confirm that the identified loss-of-function mutation in the *305_aat* variant is responsible for the lack of conversion of D3R into nasunin, we complemented the *SmAAT* function in 305E40 and DR2, two D3R-producing genotypes and homozygous for the *305E40_aat* allele (Table 2.3). To this end, a construct overexpressing the *SmAAT* cDNA coding sequence under the constitutive promoter 35S was used to obtain transformed p35S::SmAAT 305E40 and p35S::SmAAT DR2 plants.

Table 2.3 Concentration in the peel (mM, dry matter) of D3R and Nasunin (NAS), and percentage of NAS over the total (D3R+NAS) amount of anthocyanins in fruits of lines '67/3', 'DR2', '305E40' and 11 independent T1 plants transformed with p35S::SmelAAT. (Ctr, untransformed control; a, analyzed by RT-qPCR). b, data gathered from24 (Toppino et al, 2016). For each column at least a common letter indicates not different significant difference among the 'DR2' genotypes including overexpressing T1 and 'DR2' Ctr plants (Tukey test, p< 0.05).

Genotype		D3R	± sd	NAS	± sd	NAS
		mM		mM		%
DR2	Ctr	37.41	4.00 b	0.00	0.00 f	0
DR2	14-1	8.65	0.75 ef	0.29	0.42 f	2.91
DR2	18-1*	9.12	0.64 ef	7.46	0.45 b	45.03
DR2	3-1[a] *	43.96	1.28 a	3.20	0.05 de	6.78
DR2	35-1	20.94	0.25 c	5.88	0.14 c	21.91
DR2	49-1[a] *	14.66	1.84 d	2.18	0.27 e	12.93
DR2	5-5	6.74	0.15 fg	3.88	0.32 d	36.52
DR2	51-1	3.95	0.17 gh	0.04	0.03 f	0.93
DR2	6-3	1.38	0.16 h	0.11	0.02 f	7.61
DR2	8-1[a] *	12.63	1.22 de	3.14	0.51 de	19.85
DR2	8-2	25.12	0.70 c	5.22	0.34 c	17.19
DR2	32-2[a] *	23.32	1.59 c	9.18	1.02 a	28.21
305E40	3-2	9.07	0.24	2.97	0.03	24.67
305E40[b]	Ctr	12.69	0.27	0	0	0
67/3[b]	Ctr	0	0	3.28	0.05	100

A total of 26 T1 plants derived from 13 independent T0 transformation events were obtained. Twelve T1 lines (Table 2.3) were analyzed and different amounts of nasunin were detected showing that overexpression of *SmAAT* could partially restore the D3R to nasunin conversion in both DR2 and 305E40 genotypes. Three 'DR2' overexpressing plants accumulated, in the analysed fruits, very low amounts of Nasunin which were statistically not different from that of 'DR2'. In the fruit peels of the remaining eight plants a noticeable accumulation of NAS was detected, similar to that previously found in '67/3', the donor line of *SmAAT*. With regards to D3R accumulation, all the

‘DR2’ overexpressing lines accumulated significantly less D3R than the control with exception of the plant DR2 #3-1. The percentage of NAS reached a maximum of 45% of the total anthocyanin content in the DR2 #18-1 plan.

The lacking of complete conversion of the D3R into Nasunin in the transformed plants in both the genotype used, could be due to the efficiency of the transgenic protein or the *p35S::SmAAT* expression level.

Five *p35S::SmAAT* T1 lines, selected according to their different Nasunin content (highlighted with asterisk in table 2.3) were used for RT-qPCR analysis to evaluate the expression levels of the AAT endogenous and exogenous transcript in fruit peel tissue. Two sets of primers were used, (detailed in table 2.1) able to discriminate between the endogenous transcript (indiscriminately *SmAAT* or *305E40_aat* alleles) from the transgenic p35S::SmAAT transcript by using the difference between the 3’UTR region (detailed in figure 2.5).

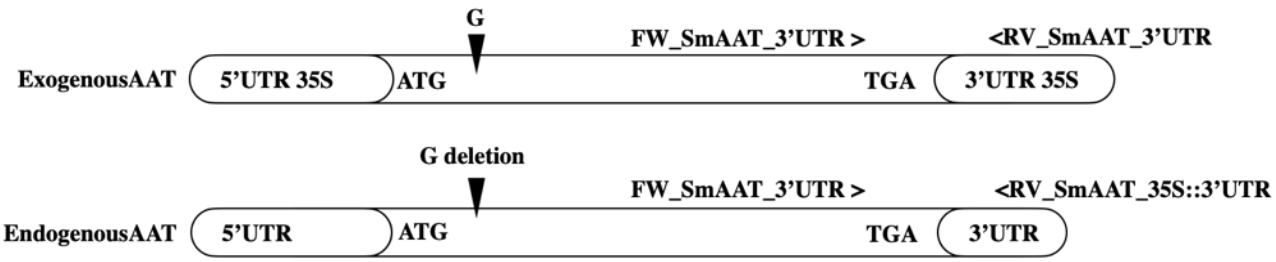

Figure 2.5: detail of the strategy utilized for primer design within the AAT sequence: the two couple of primers able to discriminate between the endogenous transcript from the transgenic p35S::SmAAT one. For this use, a common forward primer was placed on the last exon of the AAT gene while specific reverse primers were positioned on the 3'UTR region of the endogenous gene or the 3'UTR of the p35s::AAT construct, respectively. As the G deletion is not positioned within this region, it is not possible to discriminate between SmAAT and 305e40_AAT forms with these primers.

Interestingly, the expression levels of the endogenous transcripts *SmAAT* (in 67/3) and *305E40_aat* (in DR2) were not significantly different (Fig. 2.6A), suggesting that, despite the missense causing mutation, the 305E40_aat transcript does not undergo Nonsense Mediated Degradation (Shaul et al., 2015). Alternative translation of the 305E40_aat transcript into a peptide could account for this and in silico analysis suggests that a possible alternative start site downstream of the mutation this could potentially give rise to a 299aa-long peptide, starting from methionine 154 of SmAAT. This protein, however, would lack most of the first functional domain, suggesting a hindered acyltransferase activity, as supported by the absence of NAS accumulation in homozygous genotypes for this allele.

The expression levels of *p35S::SmAAT* show variation among the 5 lines analysed (Figure 2.6A) being lower in #3-1 and #49-1, while reaching levels of expression of 63% with respect to the endogenous variant *305E40_aat* in #18-1 T1 line. Reciprocally, the expression of the endogenous

305E40_aat decreased in these transgenics lines with respect to the DR2 wild type (R=-0.76) being lowest (26% of DR2) in #18-1, which was the line showing the highest absolute level of expression of the transgene. By comparing the percentage of nasunin on the total anthocyanin molar amount (D3R+nasunin) in DR2, 67/3 and the five transformed T1 lines (Fig 2.6B) we found a linear correlation between the increasing levels of nasunin and the expression levels of *p35S::SmAAT* in the transformants (R=0.91). Therefore, increasing expression levels of the *SmAAT* exogenous transcript in a DR2 background cause a decrease of the endogenous *305E40_aat* transcript (R2=0.59) and a proportional increase of nasunin levels (R2=0.87). Taken together these results prove that the *305E40_aat* allele is causative of the D3R retention in 305E40 and DR2. However, the conversion of the D3R into NAS results incomplete and variable in the different transformant lines. Starting from the assumption that the efficiency of the exogenous AAT was comparable with that of '67/3' the endogenous AAT, three non-mutually exclusive explanations can be brought forward: (1) it was hypothesized that the total amount of the anthocyanins in the berry peel of line 'D2R' line, higher than the anthocyanin is much higher than that present in '67/3' plays an important role. Probably and it is possible that the SmAAT cloned from the '67/3' is unable to convert all the D3R accumulated in the 'DR2' line into NAS Nasunin. (2) Moreover The transgenic transcript carries 5'UTRs and 3'UTRs different to those carried by from the *SmAAT* and it is possible that this affects the efficiency of translation conversion. A SmAAT antibody was not available therefore the protein levels were not compared. (3) Although the 35S promoter is generally used for overexpression analyses, previous studies suggest it might sometimes be expressed at different levels in some tissues and developmental stages. It is possible that the expression in peel tissues is not uniform and this could result in differences of expression in portions of the analyzed samples, of which RT-qPCR analyses only show an average.

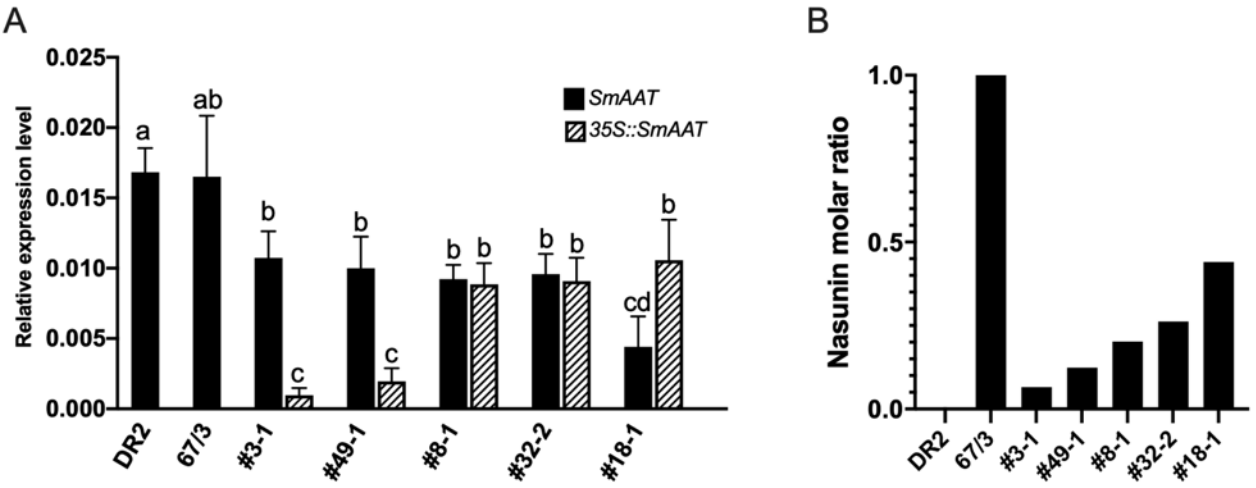

Figure 2.6 Overexpression of p35S::SmAAT in eggplant causes nasunin accumulation in the 305E40_aat DR2 genotype. (A) RT-qPCR expression analysis of SmAAT (only in 67/3 line), 305E40_aat and p35s:SmAAT in eggplants peel samples of DR2 and five T1 transformants. Data are means of three biological replicates ± SD. Oneway ANOVA with Tukey's HSD post hoc test was performed. Different letters indicate significant differences at P <0.05. (B) Nasunin mol ratio in peel of DR2, 67/3, and the five the p35S::SmAAT transformants.

The expression of functional SmAAT increases the transcription of Sm5GT1

The conversion of D3R to nasunin requires two subsequent steps of anthocyanin decoration: after the acylation of the rutinose residue with the p-coumaroyl moiety a 5-0-glucosylation takes place (Yamazaki et al., 2002). Moreover, methyltransferases enzymes have been described as capable of acting on the D3R substrate in different species: in particular *AMT* and *FMT* have been reported to be involved in this process respectively in petunia and Arabidopsis (Provenzano et al. 2014; Saito et al., 2013) The 5GT1 transferase responsible for this enzymatic step was characterized in petunia (Yamazaki et al., 2002). A homology search in the eggplant genome using the petunia 5GT1 peptide sequence as bait allowed to identify SMEL_005g238370 as the gene encoding for the eggplant orthologue *Sm5GT1* (88% identity at the AA level) a similar search in the eggplant genome using the petunia AMT peptide and the Arabidopsis FMT peptide indicated SMEL_009g331690 and SMEL_003g185620 as the unique *S. melongena* candidate orthologues of *AMT* and *FMT* respectively.

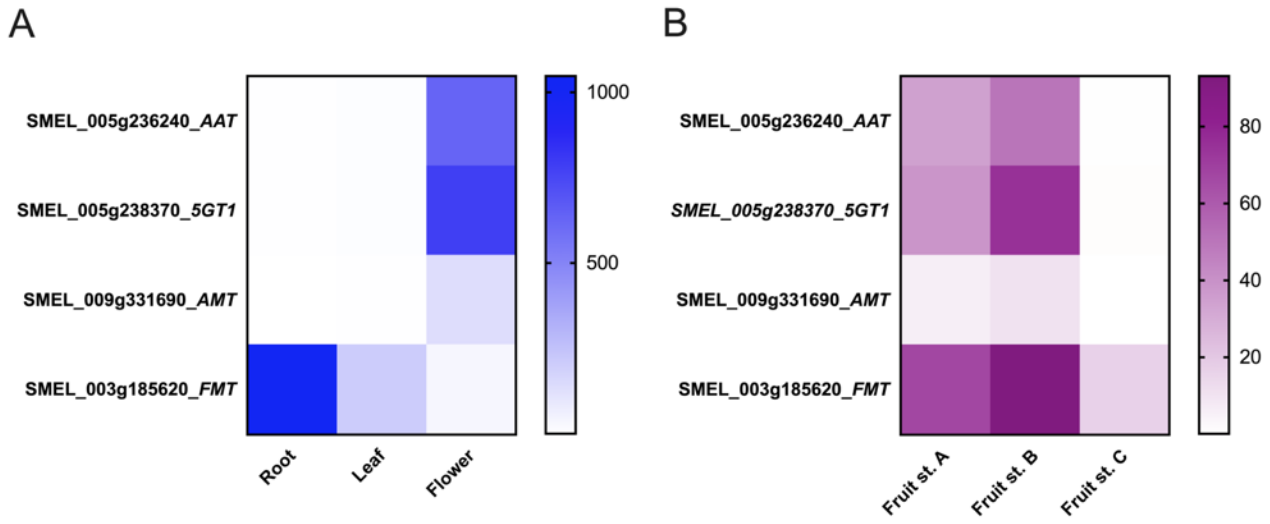

Figure 2.7: Gene expression profiles (value FPKM) of SmAAT, Sm5GT1. SmFMT, SmAMT by using the RNA-seq data of the '67/3' line (Barchi et al., 2019). A: expression in in root, leaf, flower; B: expression in fruits (stage A, B and C).

Expression levels in different tissues for the candidate genes of interest, *SmAAT*, *Sm5GT1*, *SmAMT* and *SmFMT* were obtained from previously published 67/3 RNA-seq data (Barchi *et al.*, 2019). The

relative expression levels, reported in figure 2.7, show a similar pattern for *SmAAT*, *Sm5GT1* and *SmFMT* in fruits tissue, while *SmAMT* was expressed only in flower, another pigmented tissue, and therefore included in the analysis.

As eggplant fruits at both developmental stages A and B produce anthocyanins in their peel, we investigated the expression levels of *SmAAT*, *Sm5GT1*, *SmAMT* and *SmFMT* in eggplants fruit peel of both ripening stages by comparing them in four genetic backgrounds: 67/3, 305E40, DR2 and the transformed T1 line DR2-35S::SmAAT#18-1. For *AAT* investigation, a unique set of primers was used (unable to discriminate between the endogenous transcript *SmAAT* and *305E40_aat*; Table 2.1) from the transgenic *p35S::SmAAT* transcript so that in the three WT lines 67/3, 305E40, DR2 the *AAT* expression detected could be ascribable only to the he endogenous transcript (whether *SmAAT* allele in 67/3 or *305E40_aat* in DR2 and 305E40), while the total *AAT* expression level detected in the transformed plant #18-1 is imputed to the combination of both endogenous and exogenous transcripts (demonstrated by the fact that the expression level of *AAT* resulted coincident with the sum of expressions levels of the *305E40_aat* and exogenous *35S::SmAAT* transcripts as detailed in figure 2.8. At ripening stage A (Fig 2.8A), comparable expression levels were detected for *SmAAT* between 67/3, 305E40 and DR2, while a slightly higher level was detected in DR2-35S::SmAAT#18 due to the additional expression of the *p35S::SmAAT* transcript, as observed before (Fig. 2.8 B).

At this stage comparable expression levels of *Sm5GT1* were detected among the four genetic backgrounds assayed. The expression levels of both *SmAMT* and *SmFMT*, instead, appeared much lower with respect to *Sm5GT1* and *SmAAT*, being highest in 67/3. At stage B, the expression of *SmAAT* in 67/3 resulted increased compared to 305E40 and DR2 but was comparable to that of 35S::SmAAT#18-A. Interestingly, the same expression profile was observed for the *Sm5GT1* (Fig.2.7 B) suggesting that the presence of the increased amount of *AAT* transcript due to the expression of the exogenous *35S::SmAAT* form is always associated with a correspondent increase in the expression of *Sm5GT1* in the transformed plants compared with the DR2 and 305E40 wild type genotypes. This result encourages the hypothesis that the presence of the *SmAAT* allele might bring about DR3 acylation and provide a suitable substrate for Sm5GT1, thus provoking an increase in its expression. This would be in accordance with Yamazaki et al. (2002), reporting that in petunia the acylation of the rhamnose group by SmAAT is a required step prior to 5-glucosylation. Low expression was detected for *SmAMT* with slightly higher amounts in 67/3 and the DR2-35S::SmAAT#18-A, while no statistically significant differences were observed among the very low *SmFMT* expression levels in all genotypes, (Fig.2.8 B). The differences detected for the genes *SmAMT* and *SmFMT* at stage A are unlikely related to the presence of the transgene. *AMT* was described by Provenzano et al. (2014) as involved in the determination of the flower pigmentation in

petunia, and the generally low *SmAMT* expression levels detected in the eggplant fruits peel at both ripening stages suggests that this gene is not active in this tissue, although it may still be involved in the decoration of anthocyanins in flower. Moreover, RT-qPCR analysis failed to detect significant *SmFMT* expression in eggplant peel. This result is not in accordance with previous indications from RNA-seq data (Fig. 2.7 A and B) and might be due to *FMT* expression in eggplant fruit flesh, a tissue not considered in the present work; transcriptome data suggest instead high expression in root tissues which could indicate its involvement in non-pigmented organs, and further investigation would be needed to elucidate its function.

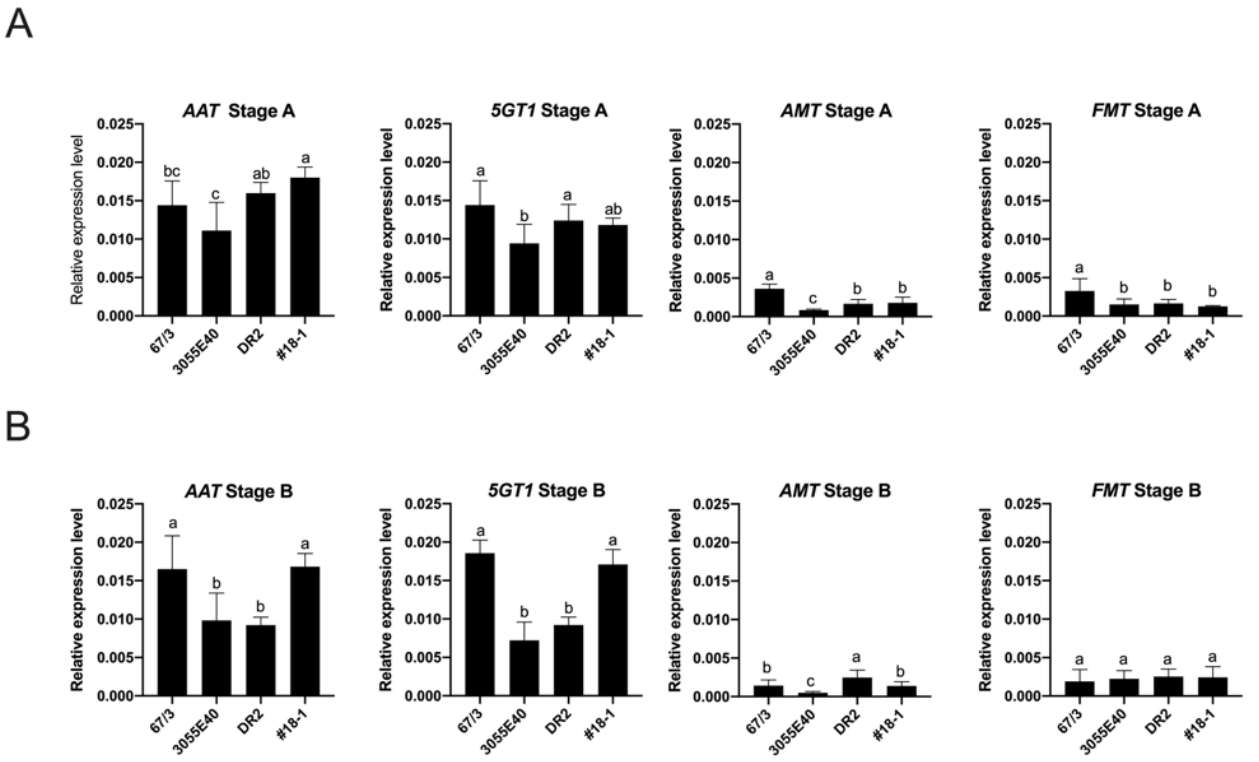

Figure 2.8: RT-qPCR based transcription profiling of eggplant AAT, 5GT1, AMT and FMT in two stages of fruit ripening (stage A and B. Expression levels, measured by RT-qPCR, are shown as relative units using SmGAPDH and Sm18S as reference gene. Data are means of three biological replicates ± SD. Oneway ANOVA with Tukey's HSD post hoc test was performed. Different letters indicate significant differences at P <0.05.

Conclusions

In light of an increasing interests towards the use of anthocyanins as alternatives to synthetic food dyes and their potential health-promoting activity, supported by evidence on their therapeutic properties (for reviews see He and Giusti 2010, Wallace et al 2016, Kalt et al 2017, Li et al 2017), in the last years there has been much scrutiny of the different forms of these pigments available from various plant sources. The peel of *S. melongena* berries accumulates high amounts of these compounds, and their chemical nature, reflected in the color they confer, differentiate the two most

common typologies of cultivated eggplant. To date, the genetic determinism of the difference between the accumulation of the anthocyanin responsible for this difference, D3R or nasunin, is still not known. In this study we identify a variant in the coding sequence of *SmAAT* as a putative mutation responsible for the difference in anthocyanin type between 67/3 and 305E40 (Fig.2.1) two accessions belonging to Type 1 "Japanese type" and Type 2, respectively. Phylogenetic analysis of sequences identified by homology and available functional data from the literature (Tohge et al., 2015) suggest that *SmAAT* encodes for an acyltransferase predicted to use D3R as a substrate (Fig. 2.2). The *SmAAT* sequence variant of 305E40 is characterized by a 1bp G deletion causing a frame shift and a premature termination of the predicted protein (Fig. 2.3); an HRM marker, designed on such a deletion, was able to distinguish the two allelic forms. Genotyping of the 67/3 x 305E40 F2 and RIL populations with the AAT-HRM marker confirmed perfect correspondence of the different allelic forms (NASU, D3R and H) with the presence/absence of D3R and nasunin. Furthermore, the genic marker proved to be strongly associated (R=0.75) with the phenotype of additional 64 accessions from the CREA-GB collection and making it a valuable predictive tool for marker-assisted breeding programs aimed at maintaining an anthocyanin type in the progeny (Table 2.2). Further confirmation for the critical role played by SmAAT in the conversion of D3R to nasunin came from the overexpression of the 67/3 cDNA sequence in both 305E40 and DR2 backgrounds, homozygous for the *305E40_aat allele*: partial nasunin levels were restored in 12 independent T1 lines (Table 2.3) confirming that the native *AAT* form is able to restore the acylation activity demanded for the conversion of D3R into nasunin. As this conversion requires a 5-O-glucosilation step in addition to the *p*-coumaryl acylation of the rutinoside (Figure 2.1), we decided to investigate whether the expression of the 5-O-glucosyltransferase putatively responsible for the completion of nasunin production was also affected in our transgenic plants. The *S. melongena* orthologue (Sm5GT1, SMEL_005g238370) of the enzyme responsible for this step in petunia (Yamazaki et al., 2002) was therefore identified by homology search in the 67/3 genome, and its expression compared to that of the *SmAAT* alleles in 67/3, 305E40, DR2 and p35S::SmAAT T1 line 18-1, in peel samples at two fruit developmental stages. The expression of the eggplant orthologues of two other genes known to encode for enzymes using D3R as substrate for metilation in other plants, *SmAMT* and *SmFMT* were also included in the analysis. Overall, the RT-qPCR results showed that partial restoration of SmAAT function was accompanied by increased levels of *Sm5GT1*, to levels comparable to those in 67/3, suggesting a substrate effect on the transcription of this gene. This is in accordance with previous reports that in petunia, 5-*O*-glucosylation does takes place only after the acylation step (Jonsson et al 1984; Yamazaki et al., 2002), and together with the absence of peaks ascribable to glucosylated forms of D3Rin the HPLC profiles of 305E40 and DR2 fruit peel, provides evidence that the same is true also for eggplant. In

addition to that, we found very low expression levels for both *SmAMT* and *SmFMT* in fruit peel, concluding they were not involved in D3R modifications in these tissues. The finding that the difference in color between type1 and type 2 accessions is due to the onset of a loss of function allele and the identified variant is the same in the majority of the tested germplasm accessions (Table 2.2) suggests that this mutation was probably selected during eggplant domestication due to human preference for fruits with different tonalities, and the mutation spread in the development of different varieties, now ascribable as type 2 (D3R-type). The fact that a limited number of accessions were not producing nasunin despite testing positive for the functional allele could be explained with at least a second, still unidentified, mutation in *SmAAT* in the germplasm. As another option, a phenotyping limitation could have contributed to this result. The visual phenotypic analysis of some genotypes resulted in fact difficult due to the extreme darkness of peel color which hampered the distinction between blackish lilac and blackish purple or in the simultaneous presence of nasunin and D3R. It is also well known that nasunin has a higher molecular weight than D3R, and considering same quantity in weight, D3R has a higher chromophore power which could cover the nasunin, if co-present. In addition, the presence of a layer of chlorophyll-producing tissue located between the peel and the outermost part of the flesh (Portis et al., 2014) could hinder the determination of anthocyanin type by means of visual analysis. Moreover, scoring for pigment type with combined co-presence of anthocyanin pigments and a chlorophyll background could prove challenging.

In conclusion we provide evidence that a single nucleotide deletion is responsible for the difference between D3R or nasunin type in cultivated eggplant and developed an HRM marker strongly predictive of the type of anthocyanin accumulated, which will be a powerful tool in marker assisted breeding programs.

Chapter 3: Insights about molecular modulation of the phenylpropanoid pathway associated to the variation of the biochemical composition of eggplant (S*olanum melongena* L.) peel at different fruit ripening stages

Introduction

Phenols are widespread compounds considered as one of the most important secondary metabolites in plant kingdom with a well-known antioxidative action associated to their healthy properties (Shadidi and Naczk, 2019; Macheix, 2017). The knowledge about these compounds in Solanum species has been mainly focused for several years on their variable content, both for qualitative and for quantitative aspects, as well as for those related to their functionality as antioxidants (Simonne et al., 2011). In the last 10 years, a special attention has been devoted within the Solanum genus especially on some edible species such as the common eggplant (*Solanum melongena* L.) for its richness in phenol compounds. In fact, eggplant berries have a high antioxidant capacity and potential healthy properties mainly due to their phenol content and composition (Akanitapichat et al., 2010). Since the first approaches, *Solanum melongena* and allied species revealed a complex phenols composition, where the most significant classes of phenol compounds have been identified as hydroxycinnamic acids, mainly represented by caffeic acid (Wu et al., 2013), with patterns of conjugation with organic acids such as quinic and malonic (Ma et al., 2010) and including also nitrogen compounds, such as amides (Whitaker and Stommel, 2003). The hydroxycinnamic acids conjugates are found in both flesh and peel of the eggplant fruit.

Moreover, another class of compounds well represented in eggplant peels are the anthocyanins, mainly as glycosidic conjugates of delphinidin, with further cis- or trans p-coumaryl derivatives (Ichiyanagi et al., 2005), so giving the blue-violet peel-pigmented genotypes in a variety of different typologies (Azuma et al., 2008). Many factors influence the qualitative and quantitative content of phenol compounds in plants, among which the ripening stage is one of the most important. In fruit and vegetables, the changes in phenolic composition during ripening has been initially extensively studied in grape, for their implication in the wine making characteristics (Delgado et al., 2004). More recently, research carried out in other fruits or vegetables such as pepper and tomato, highlighted the strong changes in the phenolic composition across the ripening stages of the fruit (Calumpang et al., 2020). In eggplant, a previous study evidenced an increased level of the total phenols, measured by the Folin-Ciocalteu method, until the maximum reached at 42 days after fruit set (Esteban et al., 1992). More recently, Mennella et al., (2012) reported a significant reduction of the two main

phenolic compounds (chlorogenic acid and the delphinidin glycosides) in the peel when fruit pass from commercial to physiological ripening stage. The process of ripening in eggplant, on the other hand, is less understood and has been investigated to a lesser extent with respect tomato and pepper. The studies so far performed on eggplant ripening have been mainly focused on the accumulation of the anthocyanin pigments, sugars, phenolics and glycoalkaloid compounds. The three main stages of unripe, commercially and physiologically ripen fruits are generally known during eggplant fruit development. In purple, violet and black eggplant fruits, the stage of commercial maturation is empirically and visually evaluated on the basis of the peel color intensity and brightness. Anthocyanin pigments accumulate to the highest levels at the unripe fruit stage and levels decline as ripening progresses. The amount of the anthocyanin coloration diminishes, and the fruit start turn from a glossy purple/lilac to a brown/dark yellow dull color when at the fully physiological ripeness (see Fig 3.1).

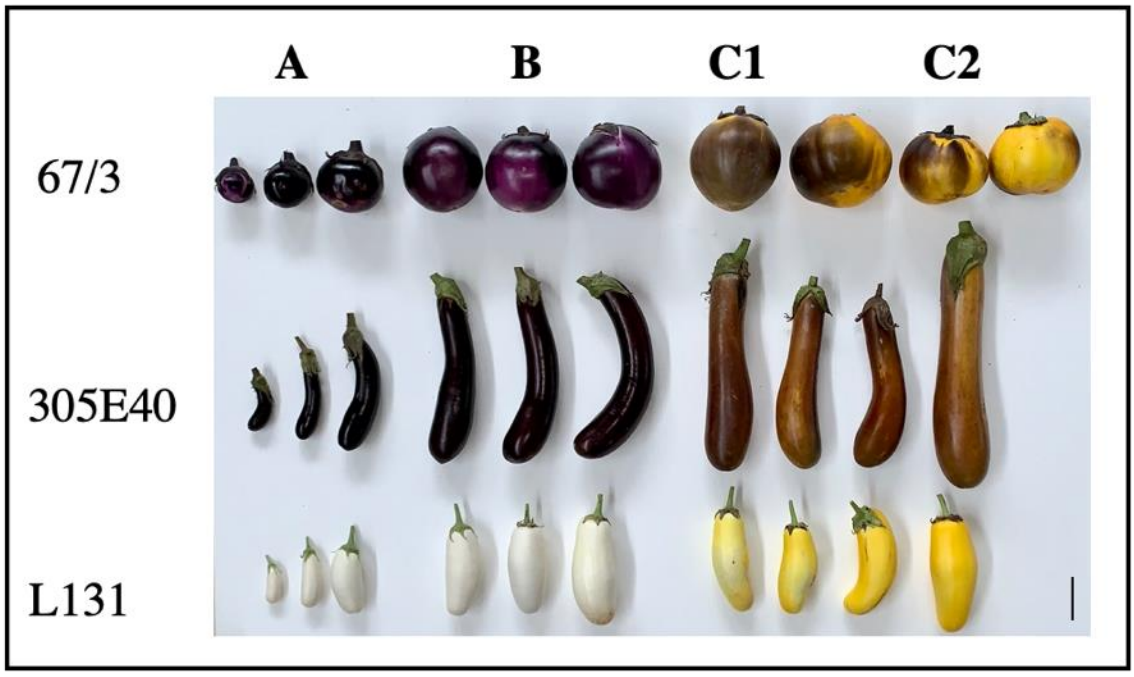

Figure 3.1 Fruits at the three stage of ripening in eggplant lines characterized by lilac (67/3), dark purple (305E40) and white (L131) peel color.

This latter ripening stage has been largely neglected in eggplant studies and there is a lack of knowledge about the molecular, genomic and metabolic changes that occur over the various stages of eggplant berry maturation. It must keep into consideration that eggplant fruit is characterized by relevant differences between commercial and physiological ripening, and over ripened fruit lose great part the crop value on the market as the consumers strongly prefer fruit at the early ripening stage, where the seed presence is not or barely detectable (Rotino et al, 1997). A recent study, aiming at evaluating the healthy properties of eggplant peel extracts for their antioxidant, anti-inflammatory

and antiviral activity, highlighted significant differences in the peel activity at the two different ripening stages of commercial and physiological ripening showing a significant anti-herpes activity was detected only in extracts from physiologically ripe peels and associated to a noticeable increase of some flavonoids (Di Sotto et al., 2018). In the present thesis, the variation of phenolic composition occurring at the late stages of maturation in eggplant peel and the possible candidate genes involved in the biosynthesis (and its regulation) of different phenolic compounds have been investigated in order to better understand such a biochemical change.

Materials and Methods

Plant material

The genetic materials employed were the two parental lines and the F1 hybrid of two F2 and RILs populations used for mapping of biochemical and phenotypical traits (Toppino et al, 2016; 2020); 305E40 (the female parent) is an introgression line from *S. aethiopicum* carrying the resistance locus *Rfo-sa1* to *Fusarium oxysporum* f.sp. *melongenae* (Toppino et al, 2008) bearing elongated dark purple fruit and containing D3R anthocyanin characterized by the presence of a green ring in the flesh next to the peel, while the male parent is the 67/3 line, whose genome has been recently sequenced (Barchi et al, 2019) and produces round lilac fruit containing the anthocyanin nasunin and white flesh; the F1 hybrid obtained from their cross is produces oblong dark lilac fruits characterized by the presence of nasunin and the green ring next to the flesh. The fourth genotype is the line L131 (Bianca Fus) which produces white fruits with white flesh and is almost deprived of anthocyanin in all its tissues and organs. Thirty plants of each accessions were grown on 2016 and 2017 seasons (May-September) at CREA Montanaso L. (45°20'N, 9°26'E) experimental farm in open field distributed in three replications of 10 plants each. Fruit harvests started in July collecting not all the commercially ripe fruits so that in August fruits were collected at unripe, ripe and overripe stages. The ripening stage was established as in Mennella et al (2012), and precisely unripe stage A, approximately 21 DAF (days after flowering), ripe stage B, (commercial stage, approximately) 38 DAF and overripe stage C, which in the present work was further divided in early (C1, approx. 60 DAF) and late (C2, approx. 80 DAF) ripeness stages. Fruits at the stage A are still actively growing, close to half of the final size, with glossy skin color, calyx, and peduncle quite tender and flexible, flesh still soft and greenish or white and seeds having not reached final size and displaying a white tegument. Fruits at the stage B have almost reached their commercial final size, with less bright skin, the flesh is less greenish and, in the 305E40 and HF1 hybrid shows the characteristic green ring next to the skin (absent in “67/3” and L131), seeds have almost reached their final size but are still

immature. The fruits harvested at the stage C have an increased firmness, the calyx and peduncle are quite lignified, the peel color turning brownish (C1) and dark brownish (C2) in 305E40 and HF1 while it turning yellowish (C1) and dark yellowish (C2) in 67/3 and L131, the flesh becomes progressively spongier with a white yellowish color containing well mature seeds. Just after sampling, the eggplant berries (10-15 for each sampling) of the three replicates were cleaned under tap water and soon prepared for the analysis by carefully trimming off the peel from each fruit (1-2 mm thinness) with a lancet. Soon after the peel samples were quickly dried in a forced-air tunnel at -50°C, and further lyophilized. After the lyophilization, the samples were powdered in a waring-blender at 4°C and stored at -20°C in glass bottles until analyses. Samples for RT-qPCR analysis were obtained by pooling tissues of eggplant peel collected from 3 plants. Very thin strips of peel were collected by using scalpel for the genotypes 67/3, 305E40 and L131 at three ripening stages (A, B and C) during the summer season 2017. All fresh tissues were immediately frozen in liquid nitrogen and stored at -80℃.

Extraction and fractionation

The initial extract was obtained by 1 g of plant powder, treated with 15 ml EtOH/HCl 0.03N 1:1. This mixture was shaken at room temperature for two hours, then centrifuged at 25000 × g at 4°C for 20 minutes. The supernatants, successively defined as "raw" extracts, have been collected by filtering on purified glass wool and stored at -80°C until use for successive analyses.

As for the preparative separation of extracts at the ripening stage C2, an aliquot of each of the genotypes (305E40, 67/3, HF1 and L131) was taken and made as a whole pool o. The solution was evaporated in the vacuum at 40°C, until the EtOH was evaporated, then four consecutive extractions were made with EtOAc saturated with water. The organic layer was separated by centrifugation and filtration on anhydrous Na_2SO4. The EtOAc was evaporated in the vacuum at 40°C, until dryness, and subsequently re-dissolved in MeOH at a volume of around 20 mL. The fractionation was performed into two steps, the first with C18 resin, the second with LH-20 resin. The first fractionation has been performed on columns (1.8 cm diameter, 5 cm height) filled with 8 g of C18 ICN Adsorbentien Biomedicals (32-63 μm, 60A) resin, preconditioned with 3 × 20 ml of MeOH 10% in water. The sample (3.5 mL), before elution, was 10-fold diluted with water, and was eluted at a flow rate of 1 ml/min. Then, the column has been subsequently eluted with 3 × 20 ml of three eluting solution at increased MeOH concentration, 30, 50 and 100%, fraction A, B and C, respectively. All resulting fractions were concentrated at small volume and re-dissolved with solutions of MeOH 50% in water, at a final volume of 5 mL.

Further separation was made by an elution on columns (1.2 cm diameter, 5 cm height) filled with Sephadex LH-20. The elution was conducted by gravity at room temperature at a flow of 0.7 mL/min and conditioned with MeOH 10% in water. Each sample from previous C18 fractions A, B and C, was 5-fold diluted with water and charged on the LH-20 columns at a volume of 5 mL. Each elution was performed with 3 × 5 ml with the following solvents, for fractions A and B: MeOH 10, 20, 30, 50 and 100%. For the fraction C the LH-20 elution was performed with MeOH 70, 100% and a solution MeOH/EtOAc 1:1. Each fraction was concentrated at small volume to eliminate the organic solvents and stored at -80°C in MeOH 50% solutions. These fractions were further analyzed by HPLC, to evaluate their composition and the effectiveness of the solid-phase separation.

HPLC analysis

The HPLC analysis was performed with a JASCO system equipped with a diode array detector (MD-2010 JASCO). The pump (PU-980 JASCO) was coupled with a quaternary gradient unit (LG-1580-02 JASCO). The analytical data were evaluated using a software-management system of chromatographic data (ChromNAV, Jasco). The separation was performed by a reversed phase, using an C18 Purospher Star 250 × 4 mm column 250 × 4 mm column. The flow rate was 0.6 ml/min, the injection volume 15µl, and the oven temperature was 42°C. The mobile phase consisted of water with 0.5 % of formic acid (solvent A), acetonitrile acidified with 0.5% of formic acid (solvent B). The gradients were as follows (A/B): 95/5 0-5 min, from 95/5 to 80/20 in 10 min, 80/20 for 5 min, from 80/20 to 55/45 in 10 min, 55/45 for 10 min, from 55/45 to 95/5 in 10 min, 95/5 for 9 min. Total analysis time was 59 min. The amount of total polyphenols (TP) was given by the sum of all the identified compounds, that were CGA, delphinidin anthocyanosides (D3R and NAS), flavonol (F), naringenin derivatives (N) and chalcones (C). Peak identification was performed both by the direct comparison with commercial standards or purified ones as described by Noda et al (2000) for anthocyanosides. Moreover, compounds mainly present in C1 and C2 stages were quantified by the calibration with analogous commercial standards: N with naringenin, C with naringenin-chalcone and F with rutin, after a carefully check of their spectral and chromatographic properties on the purified fractions and the comparison with previous literature data.

Molecular phylogenetic analysis by neighbor joining method

Evolutionary analyses of peptide sequences were conducted in MEGA X (Kumar *et al.*, 2018). The evolutionary history was inferred using the Neighbor-Joining method with a bootstrap test (500 replicates). The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. The rate variation among sites was

modeled with a gamma distribution (shape parameter = 1). All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

RNA extraction, cDNA synthesis and primer design

Total RNA from all the eggplant peel samples was extracted using the TRIzol RNA Isolation Reagents (Thermo Fisher Scientific) combined with the Spectrum Plant Total RNA kit (Sigma Aldrich), each sample was resuspended in a final volume of 30 ul. From the total RNA extracted, 8 ul were treated with the RQ1 RNase-Free DNase Kit (PROMEGA). The single strand cDNA was synthesized from 1 µg of RNA using the ImProm-II™ Reverse Transcription System Kit by PROMEGA. The standard procedure was changed by adding both 0.5 µg/reaction of Oligo(dT) and 0.5 µg/reaction of Random Primers to 1 µg of DNase treaty RNA and heating at 70 °C for 5 min. The RNA mix was cooled to 25 °C and 15 µl of RT Master mix (according to the manufacturer's instructions) was added, followed by heating to 42°C up to 1hrs and 75 °C for 10 min to inactivate the Reverse transcriptase. Primers for RT-analysis of *SmCHS_ch00, SmCHS_ch05, SmCHI_ch10, SmCHI_ch0, SmGT_ch01, SmGT_ch05, SmGT_ch10, SmDFR_ch00 and SmFLS_ch04* detailed in Table 3.1 were designed basing on the available genome sequence of 67/3 utilizing the Primer 3 software (https://bioinfo.ut.ee/primer3-0.4.0/primer3/).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) setup.

The RT-qPCR reactions were carried out according to the following PCR parameters: 95°C for 5 min, followed by incubation for 15s at 95°C and denaturation for 15s at 95°C, annealing for 60s at 59°C for 40 cycles, followed by elongation at 72°C for 20s. The reaction was performed using GoTaq® RT-qPCR Master Mix by PROMEGA. The reaction containing 1.0 µl of previously diluted cDNA (1:20), from 0.2 µl to 1.0 µl of primers (1 µM each), 5 µl of GoTaq® RT-qPCR Master Mix and RNase-Free water up to the final volume of 10 µl. All samples were run in three technical replicates, and no-template controls were included in all analysis. Standard curves for each primer pair were calculated across a 5-fold dilution series of pooled diluted cDNA amplified in technical triplicate. The PCR efficiency was calculated by Rotor-Gene 6000 Series Software and it was optimized to be in the range 90-100% with R2-values of 0.996. The expression levels of the target genes were determined in all samples in triplicates, and relative expression ratio was calculated using the "Delta-delta method" using *SmelGADPH* (Glyceraldehyde 3-phosphate dehydrogenase) and *Smel18S* as housekeeping genes (Barbierato et al 2017). Specificity of amplifications was assessed first by PCR

for the presence of a single band and then through the melt curves analysis. The analyses were performed on three biological replicates and in technical triplicates.

Table 3.1 List of primers used in this chapter.

Gene	Sequences	Scope
SmCHSch00	FW 5'- tctgcaaaacaaggcttacc -3'	RT-qPCR
	RV 5'- ataaaggcagcccattaagc -3'	
SmCHSch05	FW 5'- tcagccaaagaagggcttag -3'	RT-qPCR
	RV 5'- gttacatagataatcaattaagt -3'	
SmCHIch10	FW 5'- gtggccgaaaattgtgttgc -3'	RT-qPCR
	RV 5'- tgggaaggtctcactctgga -3'	
SmCHIch05	FW 5'- gatcaaaggttcacagtatg -3'	RT-qPCR
	RV 5'- tactagaagtagcagggaag -3'	
SmGTch01	FW 5'- gagctaaagcttataaggaa -3'	RT-qPCR
	RV 5'- gcaaacacacagaatagaaac -3'	
SmGTch05	FW 5'- gagctaaagcctataaggag -3'	RT-qPCR
	RV 5'- acagtatgttagtgactatatg -3'	
SmGTch10	FW 5'- ggctttgtgtcacactgtgg -3'	RT-qPCR
	RV 5'- gtcacttttggatcctttctg -3'	
SmDFRch0	FW 5'- ttcatcacacctacgtttccacc -3'	RT-qPCR
	RV 5'- gctcatacaggaatatatgagcc -3'	
SmFLSch04	FW 5'- cagcagcgtcttgatcatcc -3'	RT-qPCR
	RV 5'- ccaccacgacgatatccacct -3'	

Statistical analysis

Statistical analysis both for expression and biochemical data was performed with JASP software (https://jasp-stats.org/) and the graph was realized whit GraphPad Prism 9. Oneway ANOVA with Tukey's HSD post hoc test was performed. Different letters indicate significant differences at $P < 0.05$. The data are means of at least three biological replicates.

Results and Discussion

Phenolic composition of peels

The peel composition of the stage A, B, C1 and C2 with regard the TP, CGA and the anthocyanins showed a variation across the ripening stages (Table 1.1) like that reported in a previous work (Mennella et al 2012). In accordance with this and taking in consideration the transition from commercial (stage B) to the physiological (stage C1 and C2) ripen fruits, the content of CGA and especially of anthocyanins decreased dramatically in all the assayed genotypes (Table 3.2). The quantitative data related to the single assayed genotypes revealed that the main phenol compound in stage A and B, as expected, was the CGA (Table 3.2). It had the maximum values in stages A and B (664.5 and 624.7 mg/100g dw), with a decrease to 339.2 and 246.5 mg/100g dw in C1 and C2 late ripening stages, showing an average percent decrease of 62 % between stages A and C2. To resume the previous results and have a global vision of the variation, highlighting the differences over the ripening stages for the different classes of phenols analyzed a statistical analysis was performed on the data set of the average value from the four genotypes of eggplant. As for TP, CGA and ATH, the evaluation made on the calculated averages gave the same conclusions as those considered for the single genotypes, with significant higher values in stages A and B (Figure 3.2). The delphinidin anthocyanosides D3R and NAS, showed an average content in ripening stages A and B of 745.7 and 548.7 mg/100g dw, respectively, dropping to an average of 139.8 mg/100g dw in C2 stage, with an overall percent decrease of 80 % (Figure 3.2). The total content of phenols (TP, Figure 3.2) averaged at 1512.5 and 1119.8 mg/100g dw at stages A and C2, respectively, having a percent decrease of 18 % . As the clear minor decrease in the total phenol content along the ripening stages is not collinear with respect to the strong reduction affecting the two main phenolics (CGA and anthocyanins, therefore, it can be hypothesized the contribution of other phenol compound(s) which increased or were newly formed specifically in the late ripening stages. Previous works on the same environment, with eggplant of similar genotypes, found, at a stage B of ripening, levels of CGA of 1482 mg/100g dw in whole eggplant fruits in 2005-2006 samplings (Mennella et al., 2010; Mennella et al., 2012), and much higher in stage A (2319 mg/100g dw). Moreover, levels of anthocyanins resulted of an average of 378 mg/100g dw for D3R in 2005 and 2006 samplings on stage B (Mennella et al., 2010), and 1274 - 539 mg/100g dw in stage A for D3R and NAS, respectively (Mennella et al., 2012). A previous work (Whitaker and Stommel, 2003), with a robust quantitative data evaluation, found a wide range of phenols hydroxycinnamic acids in eggplant fruits, with a range of 237-1474 mg/100g dw in different fruit samplings of different commercial eggplant varieties. The here presented data are in this given range. A wide range of ATH concentrations was found in previous papers, reviewed by Nino-Medina et al. (2017), from few to high milligrams contents, from 52 to 1975 mg/100g dw in eggplant peels.

	A		B		C1		C2		
67/3	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	P value
TP	1876	134	1779	444	1531	81	1257	259	ns
CGA	970 a	50	1087 a	259	855 ab	34	343 b	27	*
NAS	660 a	81	379 ab	160	323 ab	89	145 b	39	*
305 E40	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	P value
TP	2047	310	1482	459	890	42	1101	116	ns
CGA	567 a	33	318 b	44	227 b	7	247 b	9	***
D3R	1089 a	184	944 a	379	267 b	91	170 b	20	*
HF1	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	P value
TP	1284	245	1108	279	486	65	1110	99	ns
CGA	534 a	102	500 ab	82	110 c	21	231 bc	14	**
NAS	488 a	73	324 ab	132	88 b	39	105 b	25	*
L 131	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	P value
TP	841	124	943	97	nt	-	1011	42	ns
CGA	587	9	593	186	nt	-	165	14	ns

*Table 3.2 Phenol composition of eggplant peels of four genotypes at different ripening stages A, B, C1 and C2: TP, total phenols; CGA, chlorogenic acid; D3R or NAS, delphinidine-3-rutinoside (D3R) in 305E40 or nasunin (NAS) in 67/3 and both in HF1, while no anthocyanins are detected in the white L131 accession. Each value is the average of three distinct measurements. Oneway ANOVA with Tukey's HSD post hoc test was performed. The p value significance: ns (no significant), p>.05, *p<.05, **p<.01, ***p<.001.*

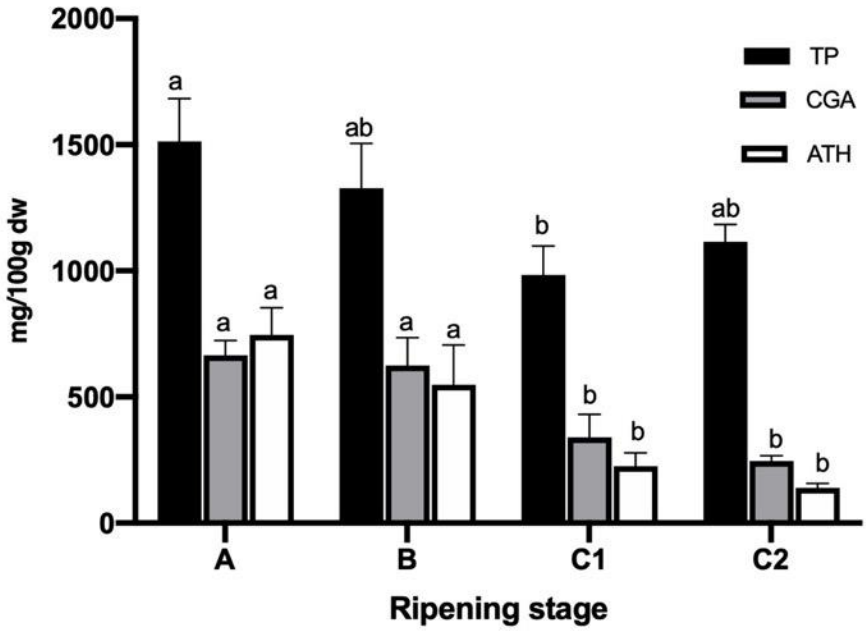

Figure 3.2 Average values of total phenols (TP), chlorogenic acid (CGA), anthocyanins (ATH) in eggplant peels of 4 genotypes at four different ripening stages (A, B, C1, C2). Oneway ANOVA with Tukey's HSD post hoc test was performed. The error bars represent the values of standard error, and different letters are for significant differences within the same class of compounds (p < 0.05).

Phenolic composition in stages C1 and C2

A correspondent and significant change in the phenolic composition took place in all the genotypes tested when fruit entered in the physiologically stage of maturation C1 and C2. In fact, the composition of the

eggplant peel extracts sampled at the full ripening stage C2 resulted in a well detectable presence of a mixture of some additional compounds than those usually found in eggplant peel in the stage A (unripe) and B (commercial ripeness), such as CGA, D3R and NAS. Interestingly, these additional compounds were particularly characteristic of the later ripening stages, with ~~no or~~ very low detectable presence in peel of immature berries extracts.

The identity of these compounds was assessed by the HPLC-RP-DAD chromatography of a semi-purified extract, where the main components resulted detectable at 280 and 355 nm. At 280 nm (Figure 3.3, top), the only clearly identified compound, by the overlapping of its retention time with that of a commercial standard, was the naringenin (naring). Other two detectable components (N1 and N2) resulted of the same family of flavanones like naring as confirmed from their UV-VIS spectra (Figure 3.4) and most probably are conjugated with polar compounds (likely glucosides), that provoke lower retention times than naringenin. The compound named as N2 resulted in a remarkable presence with respect to the other two N1 and naringenin.

The chromatogram registered at 355 nm (Figure 3.3, bottom), revealed the presence of the main peak eluting at around 36 minutes. Due to its UV-VIS characteristic spectrum, peaking at around 368 nm (Figure 3.4), and because perfectly overlapping with an extract of tomato peel with the same UV-VIS spectrum, this compound may be likely assigned as the naringenin-chalcone (nar_calc). Other three compounds, indicated in the figure 3.3 as C1, C2 and C3, and eluted before nar_calc, because probably conjugated by polar compounds, were tentatively identified as chalcones due to their UV-VIS spectra (Figure 3.4), with C2 and C3 at very close retention times. Finally, a compound, eluting at around 29 minutes (Figure 3.3, bottom), with an UV-VIS spectrum peaking at 355 nm (Figure 3.4), was tentatively identified as a flavonol-type and named as F1. These results are in accordance with those of a previous investigation by Wu et al. (2013), where some chalcones, flavanones and flavanols were found in the metabolomic profile of eggplant berries collected at ripening stage A from several accessions, performed with a new LC-MS approach.

The C_{18} and the LH-20 separation, aimed at obtaining concentrated and pure compounds available for further identifying steps, were able to obtain four fractions with the most relevant compounds in eggplant peel at late ripening stage, namely.

- compound N2, found in fraction B eluted in MeOH 30% on LH-20 resin, having an UV-VIS spectrum very close to that of naringenin and later (unpublished data) assigned as nar_glu (Figure 3.5)
- compound F1 found in fraction B eluted in MeOH 50% on LH-20 resin, having an UV-VIS spectrum related to a flavonol type compound (Figure 3.6);
- compound C2, found in fraction B eluted in MeOH 100% on LH-20 resin, having an UV-VIS spectrum very close to that of naringenin-chalcone (Figure 3.6);
- naringenin-chalcone (nar_calc), found in fraction C eluted with MeOH/EtOAc 1:1, with an UV-VIS spectrum fully close to that of naringenin chalcone (Figure 3.7).

After the identification of the main eggplant peel phenylpropanoid compounds at the late ripening stage, the semi-quantification of the three identified flavanones and the flavonol-type compounds [i. e. the N1, N2, naring and F1 (FLAV)] and the four identified chalcones, [C1, C2, C3, nar_calc (CALC)] was performed in order to characterize the changes in phenol composition correspondent to the different ripening stages in the four assayed genotypes.

The comparison of FLAV and CALC compounds, characteristic of C1 and C2 ripening stages revealed a clear opposite trend with respect to CGA and ATH, with significant higher values in C2 and C1 stages (Table 3.3). Interestingly, the levels of nar_calc and nar_gluc followed this trend, while naring showed no significant variation along the ripening stages (Figure 3.8).

The FLAV and CALC content was subjected to an increase when the fruits were at the physiological ripening stages, with the highest values of 703.7 mg/100g dw for FLAV in 67/3 at stage C2 and 296.2 mg/100g dw for CALC in the genotype L131 at stage C2 (Table 3.3).

The mean quantitative data (Figure 3.8) showed a big variability among assayed genotypes and gave for FLAV an increase in the content from stage A to stage C2 (from 251.9 to 590.2 mg/100g dw) and a steeper increase in CALC content (from 36.1 to 177.9 mg/100g dw). Unfortunately, only qualitative data of these compounds are reported in recent literature for eggplant fruit, (Wu et al., 2013; Calumpang et al., 2020) while no supporting quantitative data are available.

	A		**B**		**C1**		**C2**		
67/3	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	P value
FLAV	208	32	290	59	332	29	704	198	ns
CALC	39 ab	12	24 b	12	21 b	7	65 a	1	*
305 E40	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	P value
FLAV	363 b	105	203 ab	35	329 ab	86	529 b	100	*
CALC	29 b	7	16 b	3	66 ab	38	156 a	25	*
HF1	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	P value
FLAV	216 b	55	256 b	72	238 b	23	579 a	108	*
CALC	46 b	30	28 b	8	50 b	20	196 a	11	***
L 131	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	mg/100g DW	±sd	P value
FLAV	221	129	321	103	nt	-	550	71	ns
CALC	33 b	16	28 b	12	nt	-	296 a	50	**

*Table 3.3: Composition in flavonols and flavanones (FLAV) and chalcones (CALC) of eggplant peels of four genotypes at the different ripening stages A, B, C1 and C2. Each value is the average of three distinct measurements. Each value is the average of three distinct measurements. Oneway ANOVA with Tukey's HSD post hoc test was performed. The p value significance: ns (no significant), p>.05, *p<.05, **p<.01, ***p<.001.*

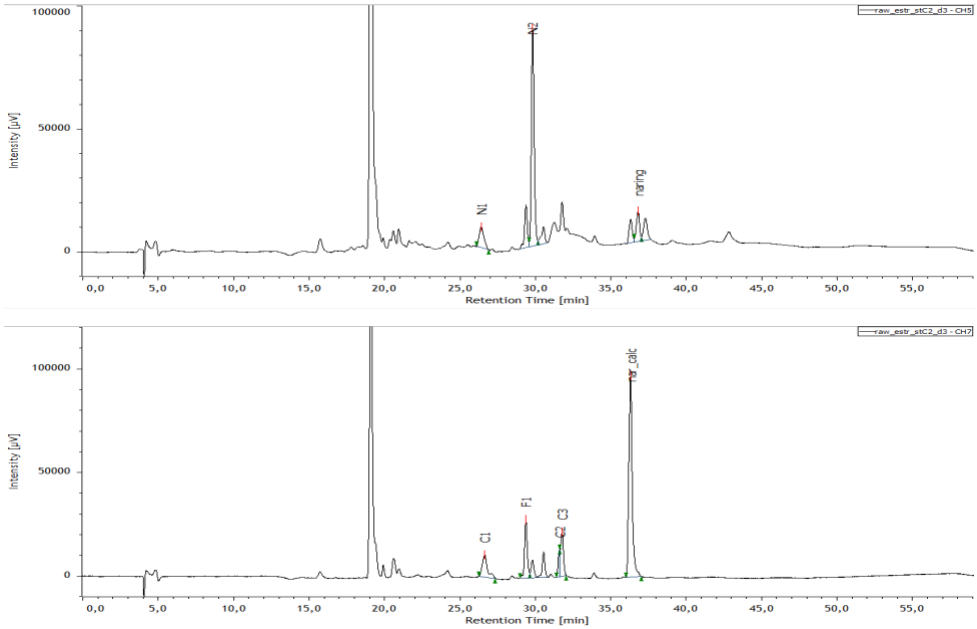

Figure 3.3:. HPLC-RP-DAD chromatogram of semi-purified extract of eggplant peel at late C2 ripening stage. The top plot is registered at 280 nm, the bottom one at 355 nm. The main identified compounds at 280 nm were indicated as naringenin (naring) and naringenin derivatives (N1 and N2). The compounds identified at 355 nm were indicated as naringenin-chalcone (nar_calc), naringenin-chalcone derivatives (C1, C2 and C3), and a flavonol type compound (F1).

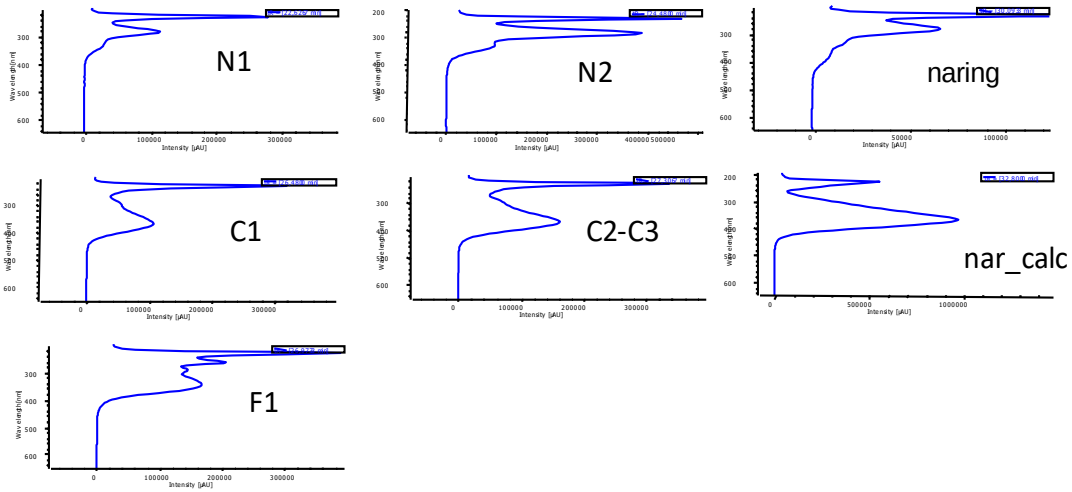

Figure 3.4: UV-VIS spectra of the separated compounds in eggplant peel extracts at late C2 ripening stage. The symbols indicate: naringenin-chalcone (nar_calc), naringenin-chalcone derivatives (C1, C2 and C3), and a flavonol type compound (F1).

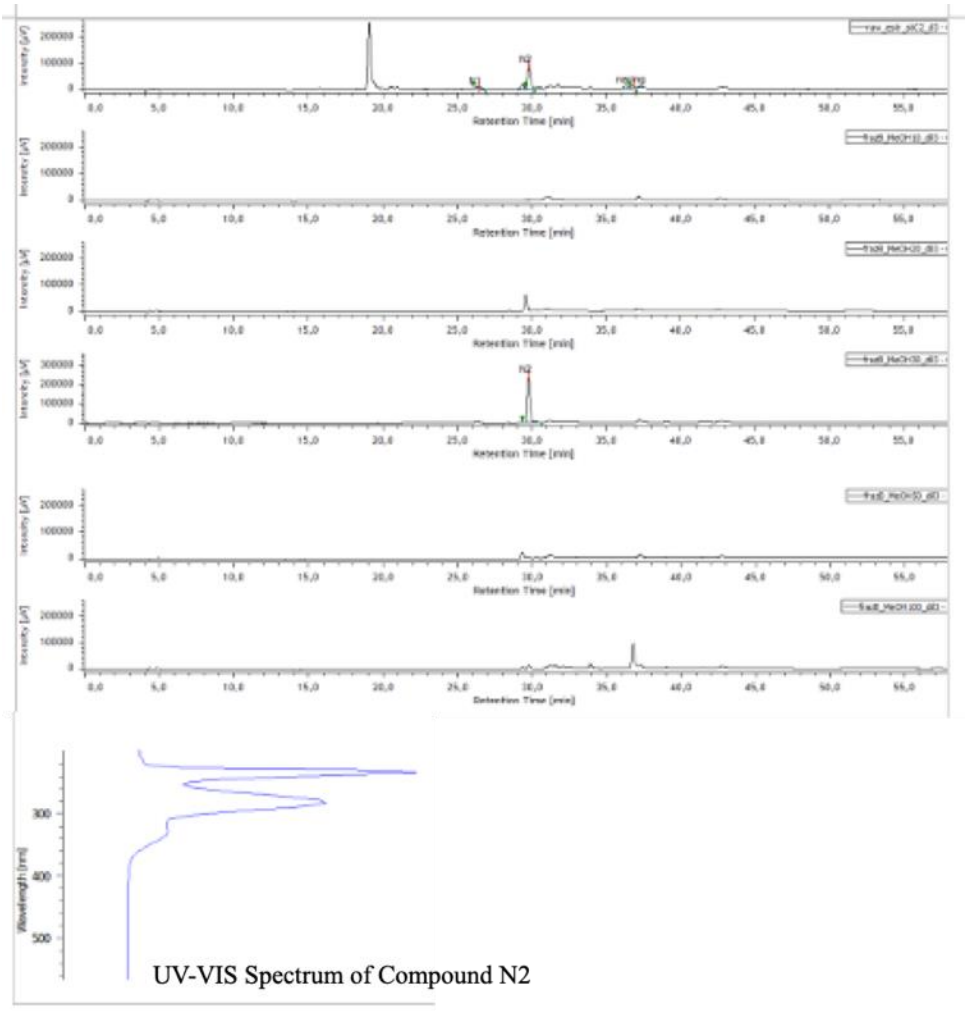

Figure 3.5: HPLC pattern registered at 280 nm of purification of fraction B, by LH-20 resin, eluting with solutions at increasing MeOH concentrations, in comparison with the initial raw extract (top chromatogram). The UV-VIS spectrum of the purified compound N2 (assigned as nar_glc) is shown at the bottom of the figure.

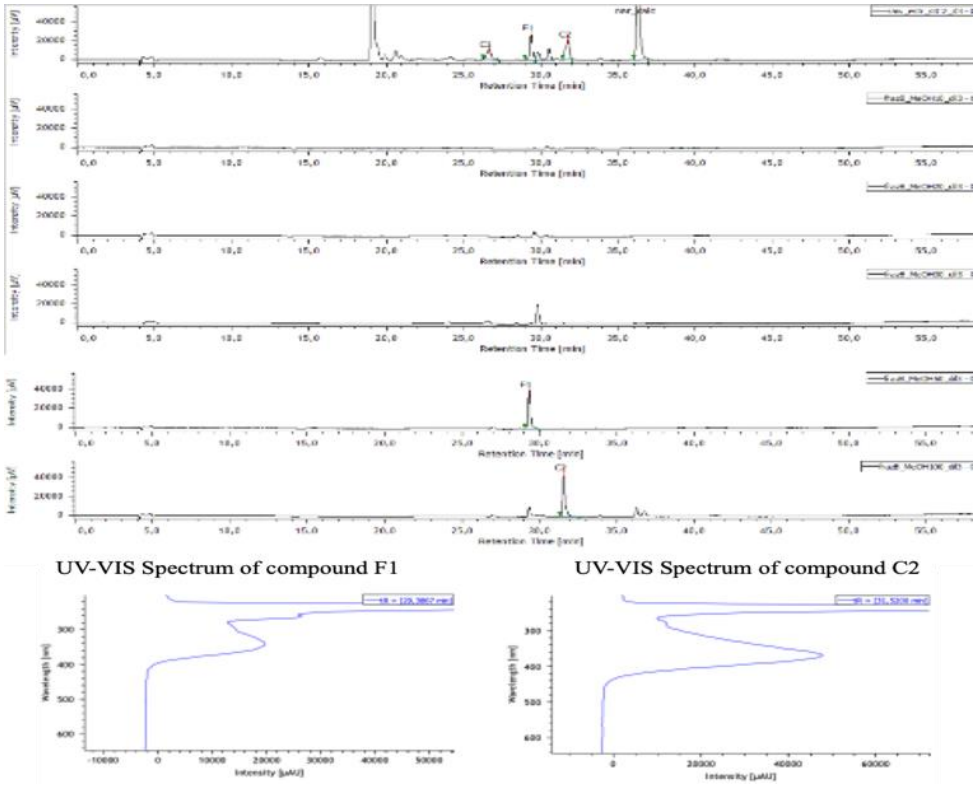

Figure 3.6: HPLC pattern registered at 355 nm of purification of fraction B, by LH-20 resin, eluting with solutions at increasing MeOH concentrations, in comparison with the initial raw extract (top chromatogram). The UV-VIS spectrum of the purified compound F1 and C2 are shown at the bottom of the Figure.

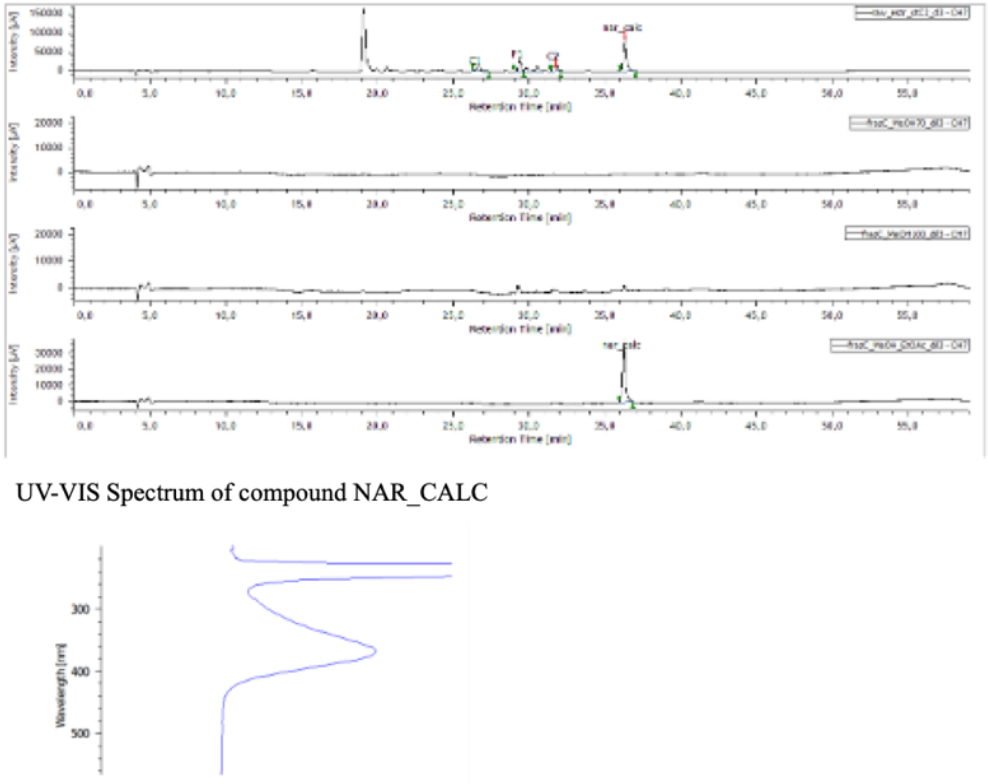

Figure 3.7:. HPLC pattern registered at 355 nm of purification of fraction C, by LH-20 resin, eluting with solutions at increasing MeOH and EtOAc concentrations, in comparison with the initial raw extract (top chromatogram). The UV-VIS spectrum of the purified compound nar_calc is shown at the bottom of the Figure.

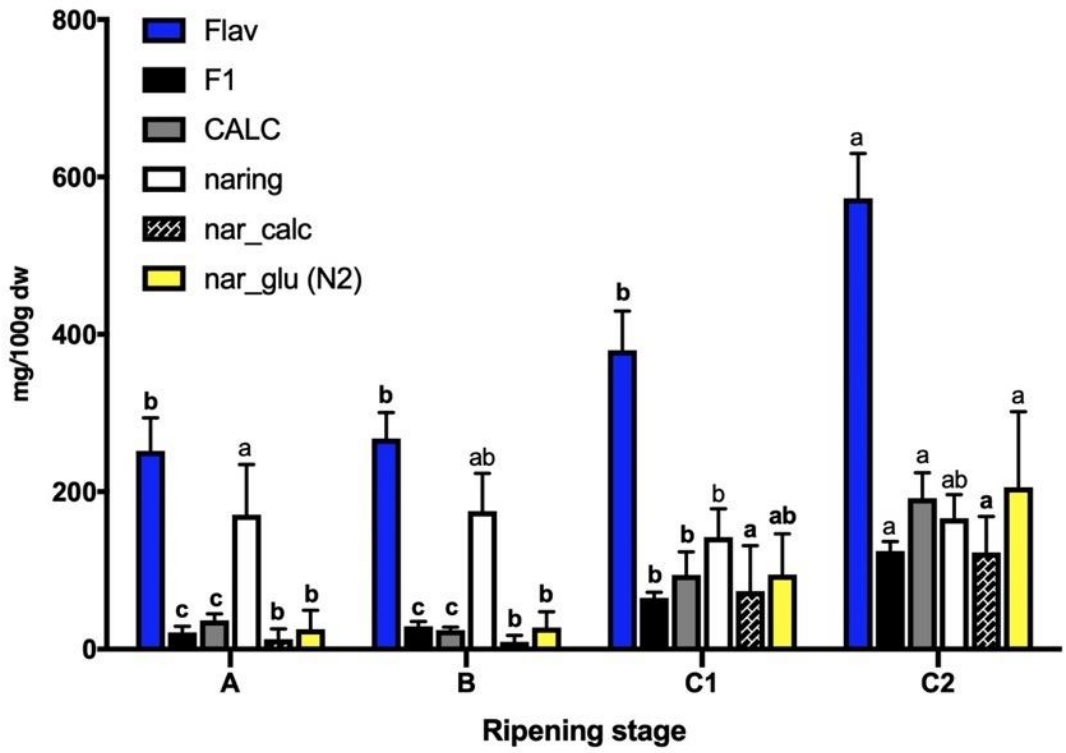

Figure 3.8: Average values of flavanones and flavonols (FLAV), chalcones (CALC), naringenin (naring), naringenin chalcone (nar_calc) and naringenin derivative (N2) in eggplant peels of 4 genotypes at four different ripening stages (A, B, C1, C2). The error bars represent the values of standard error, and different letters are for significant differences within the same class of compounds ($p < 0.05$).

Identification of candidate genes for phenylpropanoid enzymes modulated in peel during fruit ripening

To monitor the expression of genes likely involved in determining the differences in phenolic composition at different ripening stages in eggplant peel, the following candidates for key biosynthetic steps: *CHS* (Chalcone synthase) and *CHI* (Chalcone isomerase) responsible, respectively for synthesis and isomerization of the naringenin chalcone, GTs (Glucosyltransferases), potentially using naringenin as substrate, *DFR* (Dihydroflavonol-4-Reductase), involved in the biosynthesis of anthocyanins and *FLS* (Flavonol-synthase), a key enzyme for the biosynthesis of flavonols (Fig. 3.10) were chosen.

The DFR is widely reported as one of the key LBGs in anthocyanin biosynthesis in many Solanaceous species. The dihydroflavonols represent a branch point in flavonoid biosynthesis, being the intermediates for production of both the colored anthocyanins through the action of the enzyme DFR, and the colorless flavonols produced by FLS (Davis et al., 2003). To select the remaining genes, we used RNA-seq information from previous work on 67/3 (Barchi et al 2019). Two CHS genes (SMEL_005g227980, SMEL_000g090720) and two CHI genes (SMEL_010g353630, SMEL_005g226510) were chosen for analysis. To select for GT genes of interest, we first carried out

homology searches against the eggplant genome using sequences of proteins reported to catalyze the transfer of glucose from UDP-glucose to the 2′-hydroxyl group of chalcone to generate chalcone 2'-O glucoside, which confers a yellow color to the petals of carnation, cyclamen, and catharanthus (Ogata et al 2004, Togami et al 2011). All eggplant protein sequences obtained from the search with e value above e-100 were included in a Neighbor Joining analysis (Fig. 3.9) to establish their relationship with the previously described sequences. SMEL_001g119020 was selected for being close to the four carnation GT sequences and strongly expressed in fruit at stage C (Fig. 3.10) (Barchi et la., 2019), while SMEL_010g340790 was included in the analysis for its homology to catharanthus AB294401 and a discrete level of expression in fruit. Finally, it was decided to test the expression of another GT SMEL_005g234820 as a representative of a group of genes highly homologous to SMEL_001g119020 but predicted to be poorly expressed in 67/3 fruit.

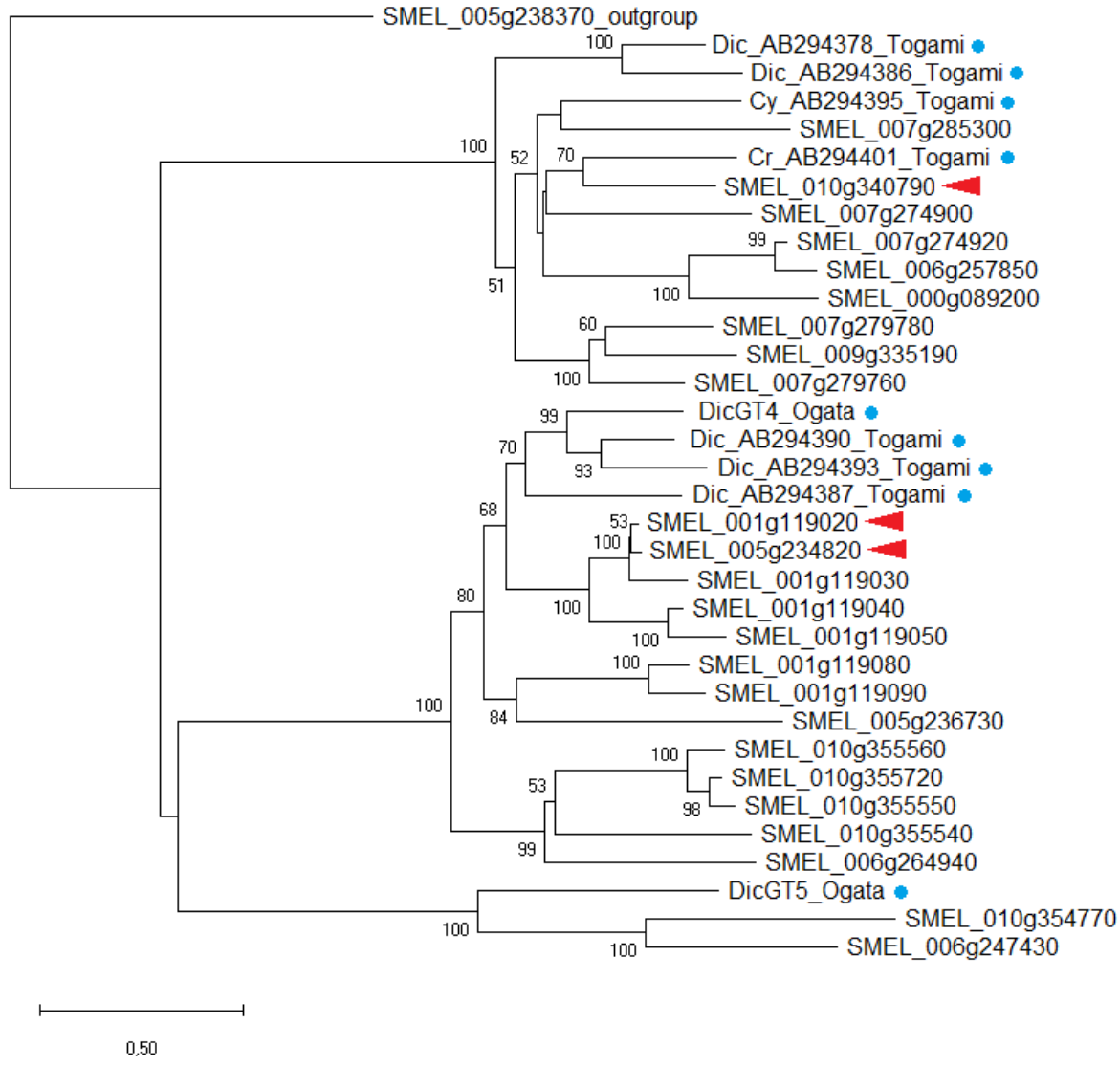

Figure 3.9. Evolutionary relationships among GT proteins of interest in eggplant. The tree was obtained using peptide sequences from carnation, cyclamen, and catharanthus reported in the literature to be involved in the formation of chalcone 2'-O glucoside (blue dots)

and eggplant sequences obtained by homology search. Red arrowheads indicate the genes chosen for subsequent RT-qPCR analysis. The sequence for a 5GT1 putatively involved in nasunin biosynthesis (SMEL_005g238370) was included as outgroup. Only bootstrap values above 50 are shown.

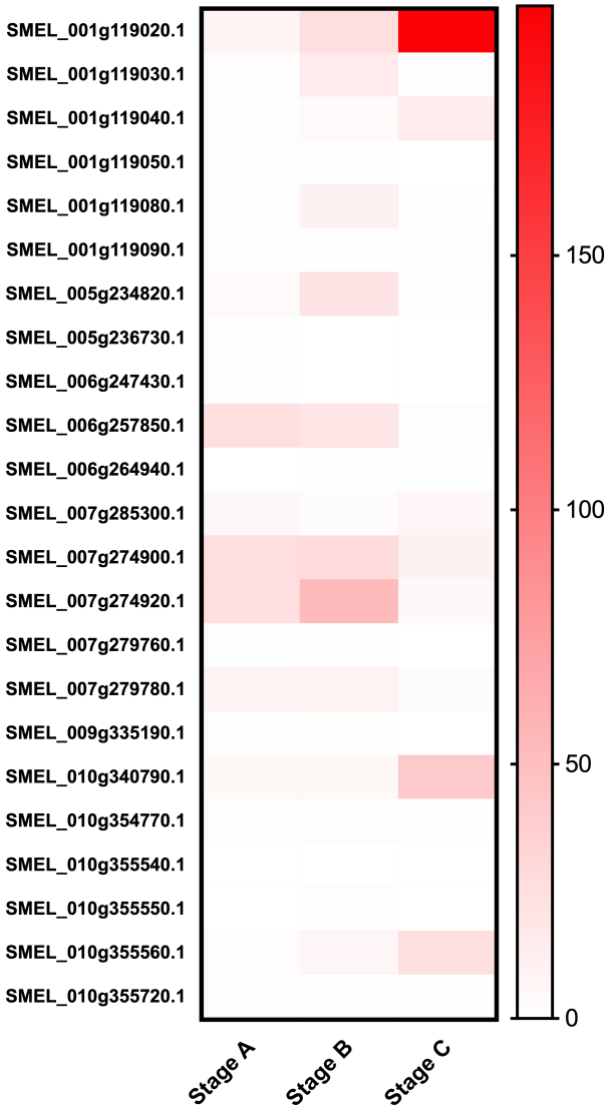

Figure 3.10: Gene expression profiles (value FPKM) of the putative SmGT identified by the phylogenetic analysis reported in Figure 3.9 by using the RNA-seq data of the '67/3' line (Barchi et al., 2019). Expression in fruits (stage A, B and C).

Transcriptional profiling of phenols related genes among ripening stages

To investigate the expression and gain insight on the potential function of the identified genes *SmCHS_ch00*, *SmCHS_ch05*, *SmCHI_ch10*, *SmCHI_ch05*, *SmGT_ch01*, *SmGT_ch05*, *SmGT_ch10*, *SmDFR_ch00* and *SmFLS_ch04*, we performed RT-qPCR expression analysis in fruit at unripe (stage A), commercial ripe (stage B) and physiological ripe (stage C). Three different genotypes were assayed: the two "pigmented" lines 67/3 and 305E40 which are characterized by the alternative production of the anthocyanin nasunin or D3R, respectively, and the "white" line L131, not producing anthocyanins. The pathway and biosynthetic steps where these genes are putatively involved are summarized in Fig. 3.11.

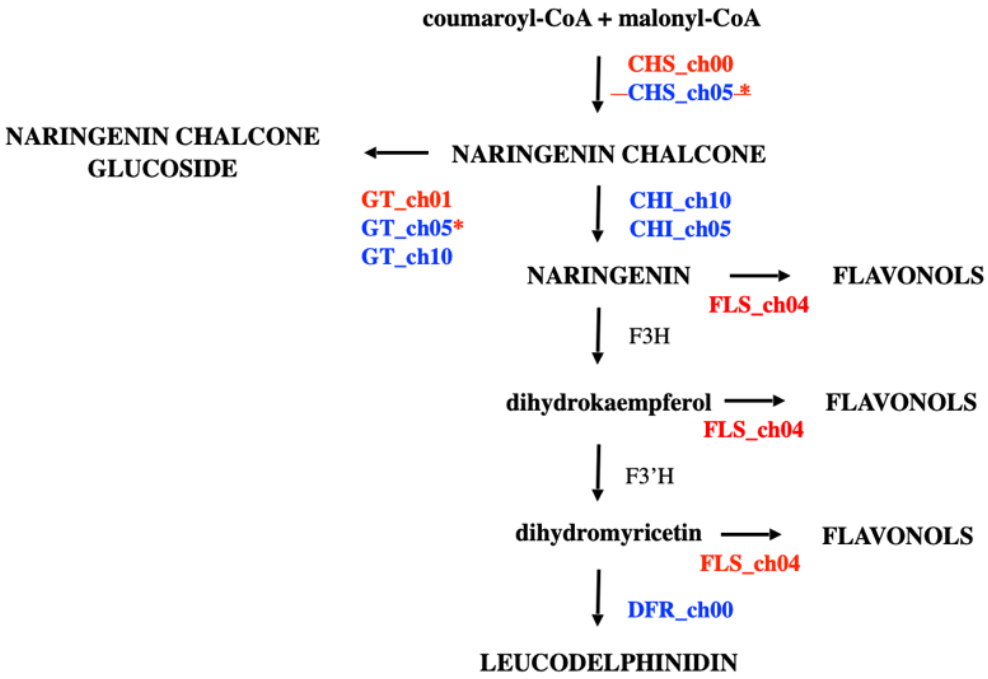

Figure 3.11: Key branch of the flavanols pathway modulated during eggplant ripening. Abbreviations of the enzymes: CHS (Chalcone synthase), CHI (Chalcone isomerase), GT (Tetrahydroxychalcone-2'-glucosyltransferase), F3H (Flavanone 3 beta-hydroxylase), F3'H (Flavonoid 3' hydroxylase), DFR (Dihydroflavonol-4-Reductase), FLS (Flavanol-synthase), a key enzyme for the biosynthesis of flavanols. The enzymes reported in red result up-regulated while those in blue are down-regulated at the ripening stage C in all the lines analyzed. The marked enzymes (*) are up regulated at the stage C only in the line "L 131".

The two *Chalcone synthases SmCHS_ch00* and *SmCHS_ch05* are putatively orthologous of genes widely described in literature to encode enzymes synthesizing naringenin chalcone starting from cumaroil-CoA and malonyl-CoA. The expression profile of these two putative genes identified show a common trend in both the pigmented phenotypes. A comparable expression level between the stages A and B and significantly higher one at stage C was detected for *SmCHS_ch00* (Fig. 3.11 and 3.12). Interestingly, the white genotype L131 as well as for the pigmented phenotypes, shows a dramatic increase in the expression level of *SmCHS_ch00 (*Fig. 3.13) at stage C. Moreover, the L131 shows the same trend also for *SmCHS_ch05,* in contrast to the pigmented genotypes which show the highest expression levels of this gene at stage A. The two homologous of *Chalcone isomerase*, *SmCHI_ch10* and *SmCHI_ch05* are responsible for the isomerization of the naringenin chalcone into naringenin. These genes result clearly expressed in the pigmented plants at stage A and not or weakly expressed at the stage B and C (Fig. 3.12 and 3.13). By contrast, in L131 a well detectable and significantly different level of expression was evidenced for *CHI_ch05* in the peel at stage C with respect to A and B stages, while the gene on ch10 had a very low expression in all ripening stages (Fig. 3.14). The lack or very weak expression of CHI in the genotype L131 may be in relation to the white peel phenotype of this line. The expression profile of the three putative *tetrahydroxychalcone-2'-*

glucosyltransferase in the pigmented genotype confirmed that the best candidate for the naringenin chalcone glucoside formation at stage C is the *SmGT_ch01* due to its high expression levels in that tissue, while the involvement of the *SmGT_ch05* (mainly expressed at stage A in pigmented fruits) and *SmGT_ch10* (barely detectable), if any, it is almost irrelevant. A similar trend to the pigmented accessions was evidenced in the L131 for *SmGT_ch01* and *SmGT_ch10*. By contrast, the *SmGT_ch05* in L131 genotype show a trend comparable to the one observed for the *SmGT_ch01*. Through the alignment of the protein sequences of *SmGT_ch01* and *SmGT_ch05* a high identity has been found, allowing to speculate that could be homologous genes which together can realize the glycosylation of naringenin chalcone. The *SmDFR_ch00* was only expressed in the two pigmented genotypes at stage A. In the L131 genotype, no expression of the *SmDFR_ch00* was detected. The *SmFLS_ch04* result strongly expressed at the stage C in all the genotypes showing an opposite trend with respect to *SmDFR_ch00* coherently to what already described by Barchi et al., (2019).

The data suggest that collectively CHS genes are expressed in eggplant fruits peel at all stages, with a peak of expression at stage C. The observed drop in expression of the *CHI* genes at stages B and C (causing a cascade depletion in the expression of the genes belonging to the same branch of the pathway and a consequent reduced production of new anthocyanins at those stage) could be predictive of a switch in the biosynthetic pathway and a consequent increase of the naringenin chalcone throughout maturation. Moreover, the expression profile of *SmGT_ch01* in all genotypes and *SmGT_ch05* in L131 is consistent with the further conversion of naringenin chalcone in its glucosylated form at the physiological ripe stage. The sharp increase of expression for *SmFLS* at stage C is suggestive of a channeling of the pathway towards the production of flavonols at mature ripe stage, which is consistent with the drop of *SmDFR* levels in pigmented genotypes after stage A. FLS belongs to the 2-oxoglutarate-dependent dioxygenase family, known to convert naringenin into 3-O-methylkaempferol (Kim et al., 2010) and to compete with F3^{1}H for dihydrokaempferol and with DFR for dihydromyricetin (Forkmann et al. 1986). To the best of our knowledge, in this thesis we report for the first time a group of genes, represented by two *Tetrahydroxychalcone-2'-glucosyltransferase* (*SmGT_ch01* and a putative paralogue *SmGT_ch05*) and the *Flavonol synthase* (*SmFLS_ch04),* which show a trend of expression strongly correlated with the ripening process in eggplant. The bibliographic data reported to describe the enzymatic activity of the candidate genes here analyzed along with the phenolic compounds identified in this work for the first time confirm that such genes are probably modulated in the evolution of the chemical make-up of eggplant peel during ripening. Interestingly, at stages A and B in the anthocyanin-less L131 genotype, no increase in the expression of the two *SmGTs* was detected, while the yellow color typical of the C stage appears coincident with the activation of these genes. This is coherent with what observed by Togami et al., (2011) who

reported a correlation between tetrahydroxychalcone-2'-glucosyltransferase activity and increasing levels of yellow pigmentation in flowers of different plant species. Moreover, as *FLS* is not expressed at stages A and B of the L131 line, suggest that likely a genotype-dependent regulation networks may occur in the *S. melongena* gene pools. Therefore, further studies are needed to identify genes involved in the formation of the colorless phenols identified in this work as well as in genotypes displaying other skin pigmentation (eg., green, striped)

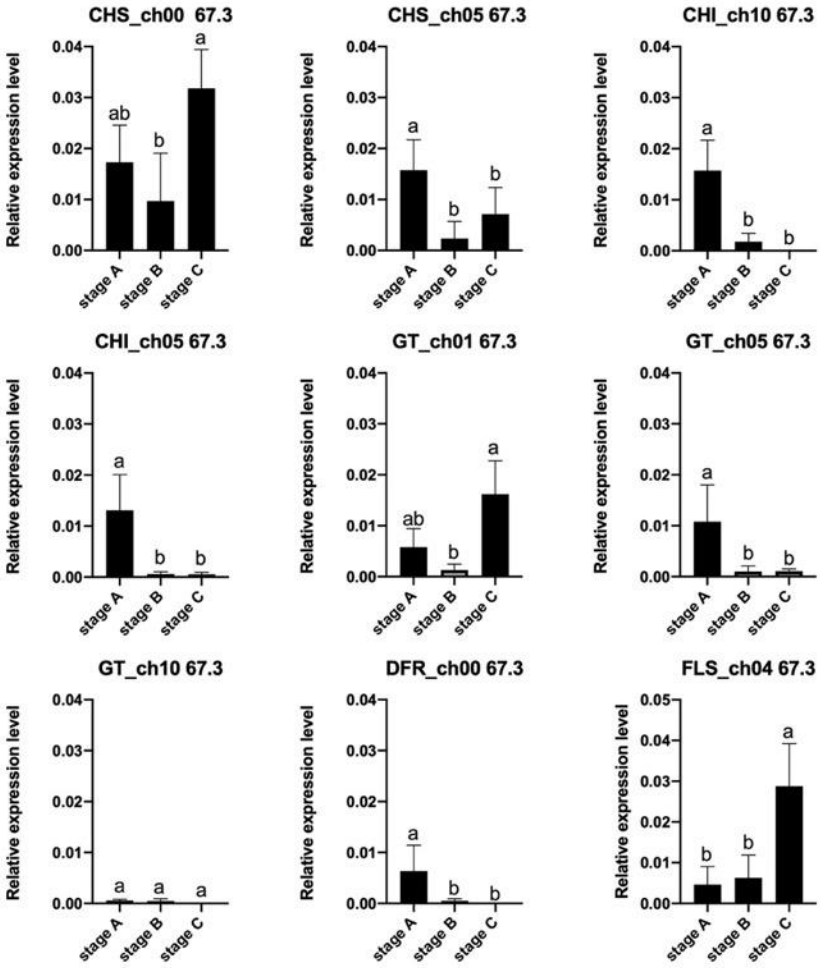

Figure 3.12: RT-qPCR transcription profiling of SmCHS_ch00, SmCHS_ch05, SmCHI_ch10, SmCHI_ch00, SmGT_ch01, SmGT_ch05, SmGT_ch10, SmDFR_ch00 and SmFLS_ch04 in the pigmented eggplant genotype "67/3" in three stages of fruit ripening: unripe (stage A), commercial ripen (stage B) and physiological ripen (stage C). Expression levels, measured by RT-qPCR, are shown as relative units using SmGAPDH and Sm18S as reference gene. Data are means of three biological replicates ± SD. Oneway ANOVA with Tukey's HSD post hoc test was performed. Different letters indicate significant differences at P <0.05.

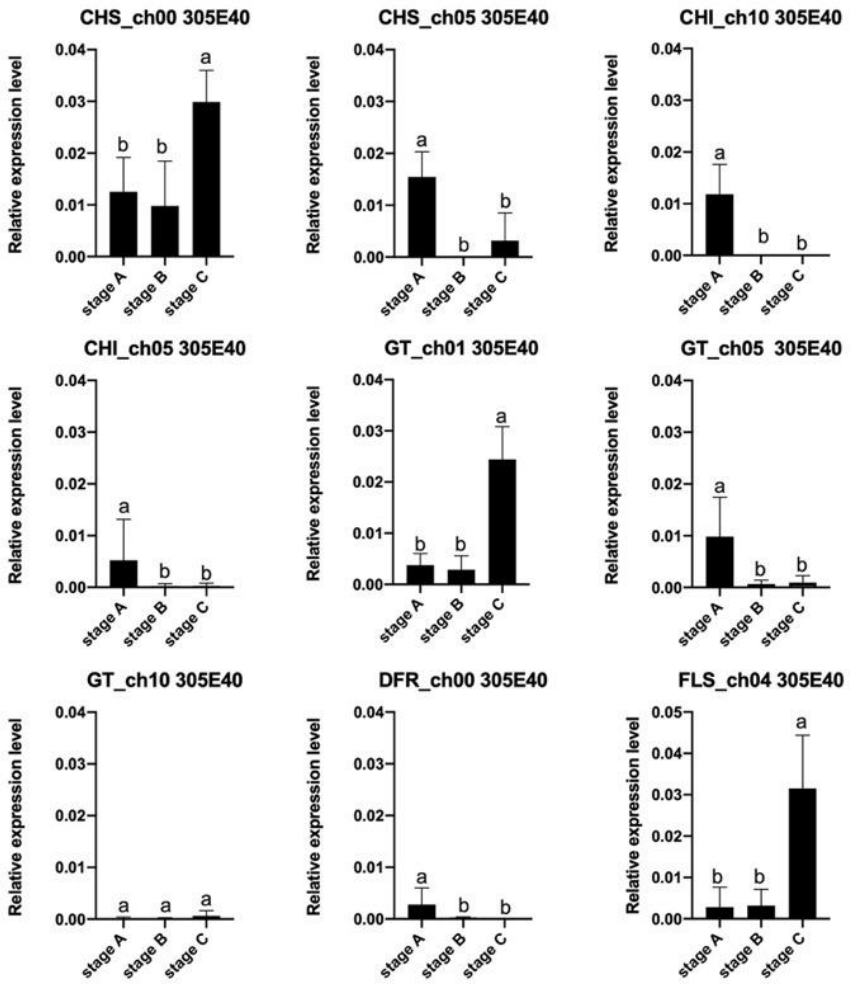

Figure 3.13: RT-qPCR transcription profiling of SmCHS_ch00, SmCHS_ch05, SmCHI_ch10, SmCHI_ch00, SmGT_ch01, SmGT_ch05, SmGT_ch10, SmDFR_ch00 and SmFLS_ch04 in the pigmented eggplant genotype "305E40" in three stages of fruit ripening: unripe(stage A), commercial ripen (stage B) and physiological ripen (stage C). Expression levels, measured by RT-qPCR, are shown as relative units using SmGAPDH and Sm18S as reference gene. Data are means of three biological replicates ± SD. Oneway ANOVA with Tukey's HSD post hoc test was performed. Different letters indicate significant differences at P <0.05.

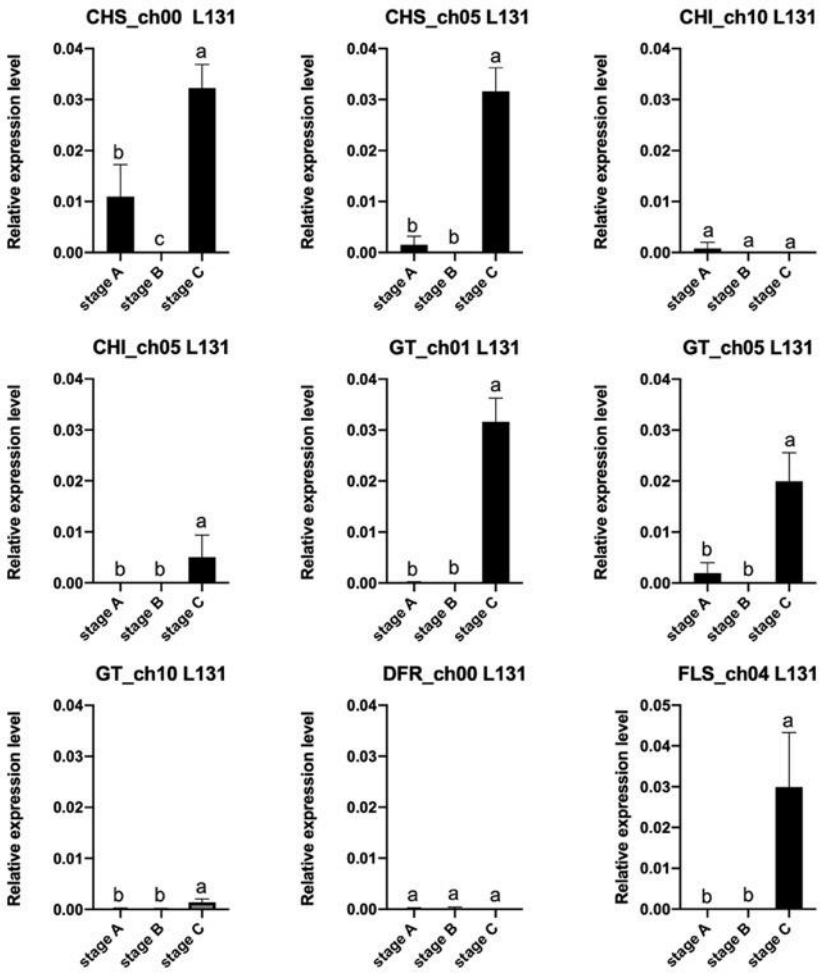

Figure 3.14: RT-qPCR transcription profiling of SmCHS_ch00, SmCHS_ch05, SmCHI_ch10, SmCHI_ch00, SmGT_ch01, SmGT_ch05, SmGT_ch10, SmDFR_ch00 and SmFLS_ch04 in the white eggplant genotype "L 131" in three stages of fruit ripening: unripe(stage A), commercial ripen (stage B) and physiological ripen (stage C). Expression levels, measured by RT-qPCR, are shown as relative units using SmGAPDH and Sm18S as reference gene. Data are means of three biological replicates ± SD. Oneway ANOVA with Tukey's HSD post hoc test was performed. Different letters indicate significant differences at P <0.05.

Conclusions

The present work definitely confirmed that the accumulation of naringenin chalcone, its glucoside conjugated and other flavonols/flavanols characterize the stage of physiologically mature fruits in *S. melongena*. Moreover, the *SmFLS_ch04, SmGT_ch01 and SmGT_ch05* are proposed as strong candidate genes underlying the enzymatic steps of phenols biosynthesis in the physiologically ripen eggplant fruits. This finding gives the opportunity to consider that the higher anti-viral activity detected in extracts from physiologically ripe peels (Di Sotto et al, 2018) may be attributable to the presence of naringenin chalcone and its derivatives. These unmarketable overripe eggplant fruit (Mauro et al, 2020), currently considered a field waste because not employable for human consumption, may be efficiently exploited in nutraceutical and pharmacological sectors. A better knowledge of the biosynthesis and accumulation of phenylpropanoids including flavanols and flavonols compounds is a breeding challenges for the improvement of this species and may have an important practical impact in its cultivation.

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General Conclusion

This Ph.D. research project is based on the molecular characterization of genes involved in the biosynthetic pathway of anthocyanins in eggplant (*S. melongena* L.) fruit and in its regulation by taking into consideration different ripening stages and genotypes displaying different phenotypes for the considered features.

In the respect of the original purposes, different hypotheses were elaborated and then validated using various experimental approaches. The results obtained are here summarized.

- Focusing on the highly conserved MYB-bHLH-WD (MBW) complex, which is pivotal in the transcriptional regulation of the anthocyanin biosynthetic pathway, it was possible identified and characterized in eggplant the genes: SmelANT1, SmelAN2, SmelJAF13, SmelAN1. Furthermore, the regulatory R3 MYB type repressor Smel-MYBL1, never reported before, was identified and characterized. Moreover, RT-qPCR analysis revealed specific transcriptional patterns of bHLH (SmelAN1, SmelJAF13) and MYBs (SmelANT1, SmelAN2 and SmelMYBL1) in different plant tissue/organs and at two stages of fruit development.
- Thought the deep characterization of the QTLs already spotted in the F2 and RIL eggplant populations from the cross 305E40 x 67/3 associated with the anthocyanin type and accumulation, two genes (*SmAAT* and *Sm5GT1*) involved in the final "decoration" steps of the anthocyanin pathway in eggplant were identified and characterized. It was proved that a single nucleotide deletion in the *AAT* gene is responsible for the accumulation of D3R or nasunin anthocyanin type in the peel of colored eggplant fruit. A HRM marker strongly predictive of the type of anthocyanin accumulated was developed which can be employed for MAS of this trait.
- In the present thesis it was demonstrated that the accumulation in the peel of naringenin chalcone, its glucoside conjugated and other flavonols/flavanols characterize the stage of the eggplant physiologically mature fruits along with the dramatic decrement of the anthocyanin content. Mirroring these biochemical changes and consistently with them a modulation of *SmFLS_ch04, SmGT_ch01 and SmGT_ch05* genes may underlying the enzymatic steps of phenols biosynthesis in the physiologically ripen eggplant fruits.