**GABRIEL LASMAR DOS REIS**

# NOVEL microRNAs CONTROLLING THE ANTHOCYANIN BIOSYNTHESIS PATHWAY IN TOMATO

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, para a obtenção do título de Mestre.

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**GABRIEL LASMAR DOS REIS**

**NOVEL microRNAs CONTROLLING THE ANTHOCYANIN BIOSYNTHESIS PATHWAY IN TOMATO**

**NOVOS microRNAs CONTROLANDO A ROTA DE BIOSSÍNTESE DE ANTOCIANINA EM TOMATE**

Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, para a obtenção do título de Mestre.

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*A Deus, a minha mãe Celma, meu pai Josué e ao meu irmão Felipe.*
*Dedico*

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# RESUMO

As antocianinas são compostos pertencentes ao metabolismo secundário das plantas derivadas do grupo dos flavonoides. Elas são responsáveis pela coloração azul, vermelha ou roxa em diferentes tecidos da planta, como folhas, flores, sementes e frutos. Nas plantas, elas são principalmente responsáveis pelo crescimento e desenvolvimento, defesa contra patógenos, tolerância a seca e proteção contra radiação UV. Além disso, as antocianinas são extremamente benéficas para a saúde humana, elas combatem e previnem uma série de doenças crônicas. Dentre estes benefícios, se destacam os efeitos antioxidantes, prevenção contra doenças cardiovasculares e câncer. Os principais vegetais e frutas presentes em nossa dieta acumulam antocianina apenas em pequenas quantidades, sendo a maioria restrita as camadas epidérmicas. Pela casca representar menos de 5% da massa total das partes comestíveis da planta, um aumento nos teores de antocianina é desejável, e o tomate se mostra um ótimo candidato para este aumento, visto que ele representa o principal vegetal consumido em todo o mundo. Os microRNAs (miRNA) são uma classe de pequenos RNAs não codificadores de proteínas, de tamanho variável entre 20-24 nucleotídeos, atuando no controle da expressão de genes codificadores de proteínas, por meio da clivagem do mRNA ou bloqueando a sua tradução. No tomate, a atuação dos miRNAs na rota de biossíntese de antocianina ainda é pouco estudada. Visando um melhor entendimento da atuação dos miRNAs nesta rota, realizamos a predição, validação e *knockout* de alguns miRNAs bem como análises de expressão para alguns genes relacionados à rota de biossíntese da antocianina. A predição de alvos para os miRNAs identificou os genes *Anthocyanidin synthase* (ANS), SlAN1 (bHLH) e *Chalcone Synthase* (CHS) sendo possivelmente regulados por miR5368, miR6024-3p e miR9471b-3p respectivamente. Analisamos a expressão destes miRNAs e dos genes relacionados a rota da antocianina no Micro-Tom *Aft/atv/hp2* e Micro-Tom Selvagem por RT-qPCR. Posteriormente, realizamos o *knockout* de cada precursor destes miRNAs no Micro-Tom Selvagem pela técnica CRISPR-Cas9. Como resultados, observamos um fenótipo roxo nas nervuras e na haste dos explantes transformados, indicando que o miR5368, miR6024 e miR9471 promovem uma regulação negativa na rota de biossíntese de antocianina atuando nos genes ANS, SlAN1 e CHS respectivamente.



**Palavras-chave:** Tomate. Biossíntese de Antocianina. microRNAs. CRISPR-Cas9.

## ABSTRACT

The anthocyanins are compounds from the secondary metabolism of plants and belong to the flavonoids group. They are responsible for the blue, red and purple pigmentation in many plant tissues, as leaves, flowers, seeds and fruits. For plants they are mainly responsible for the growth and development, defense against pathogens, drought tolerance and protection against UV radiation. In addition, the anthocyanin are extremely beneficial to human health, they fight and prevent a range of chronic diseases. Among these benefits, antioxidant effects, prevention against cardiovascular diseases and cancer stand out. The main vegetables and fruits present in our diet accumulate anthocyanin just in small quantities, and in most cases being restricted to the epidermal layers of the edible parts. Since the peel usually represents less than 5% of the total mass of edible parts of the plant, a high anthocyanin accumulation is desirable, and the tomato is a great candidate for this improvement, since it is the principal vegetable in worldwide. microRNAs (miRNAs) are a class of small non-coding RNAs that contain 21-24 nucleotides and regulate gene expression post transcriptionally, by mRNA cleavage or suppressing translation. In tomato, the miRNAs role in the anthocyanin biosynthesis pathway is still not well characterized. Aiming for a better understanding of the action of the miRNAs in this pathway, we performed the prediction, validation and knockout of some miRNAs as well as the expression analyses for some of the anthocyanin pathway related genes. The miRNA target prediction identified the genes Anthocyanidin synthase (ANS), SlAN1 (bHLH) and Chalcone Synthase (CHS) being possible regulated by the miR5368, miR6024-3p and miR9471b-3p, respectively. We analyzed the expression of these miRNAs and anthocyanin related genes in Micro-Tom triple mutant *Aft/atv/hp2* and Micro-Tom Wild-Type by RT-qPCR. Afterwards, we performed the knockout of each miRNA precursor in Micro-Tom Wild-Type by CRISPR-Cas9. As a result, we observed transformed explants with a purple phenotype in leaf veins and stem, indicating that the miR5368, miR6024 and miR9471 promote negative regulation in the anthocyanin biosynthesis pathway acting in the genes ANS, SlAN1 and CHS respectively.



**Keywords:** Tomato. Anthocyanin Biosynthesis. microRNAs. CRISPR-Cas9.

**SUMMARY**

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# PART 1

# 1. INTRODUCTION

Tomato (*Solanum lycopersicum*) represents one of the most important horticultural crops in the world and their fruits are the principal component in human diet (BORGHESI et al., 2011). This high production and consumption can be explained by its flexibility, since it can be consumed fresh or processed by the industry in different ways (FILGUEIRA, 2003). The tomato fruits contain many essential nutrients for human health, such as vitamins, lycopene, carotenoids and flavonoids (GEORGIEVA et al., 2014). The presence of some nutrients is associated with fruit color. For example, orange fruits contain carotenoids as a major pigment, the red ones contain lycopene and the purple ones are associated with the anthocyanins (FILGUEIRA, 2003). However, the commercial varieties of *Solanum lycopersicum* generally do not accumulate anthocyanin in their fruits.

Anthocyanins are compounds belonging to the flavonoid group and are one of the most studied chemical groups (GU et al., 2003). They are present in many species and are responsible for the blue, red or purple pigmentation of different plants tissues, such as leaves, flowers, seeds and fruits (HE et al. 2011; TANAKA & OHMIYA, 2008). By providing this differential to the plant tissues, anthocyanins promote the attraction of pollinators and seeds dispersers. In addition, they are related with other processes, such as growth and development, defense against pathogens, drought tolerance and protection against UV radiation (BUER et al. 2010; CHRISTIE et al. 1994; SARMA & SHARMA 1999).

In addition to the benefits it confers to plants, anthocyanin is able to provide benefits to human health by its biological activity, as antioxidant and inflammatory proprieties (DIACONEASA et al., 2015), prevention of cardiovascular disease (TOUFEKTSIAN et al., 2008), cancer prevention (BUTELLI et al., 2008; CHAREPALLI et al., 2015), visual improvement and prevention of obesity and diabetes (GUO & LING, 2015). Because of these particular properties, the use of anthocyanins has been widely demanded, mainly by the food, pharmaceutical and cosmetic industries. Unfortunately, in most of the vegetables and fruits, the anthocyanin is present just in small quantities, and in most of the cases being restricted to the epidermal layers of the edible parts (BUTELLI et al., 2008). Since the peel usually represents less than 5% of the total mass of edible parts of the plant (SESTARI et al., 2014), a high anthocyanin accumulation is desirable, and the tomato is a great candidate for this improvement, since it is the principal vegetable in worldwide.

The commercial tomato *Solanum lycopersicum* accumulates anthocyanin only in its vegetative tissues and small quantities of flavonoids as chalcone narigenin present in the epicarp of the fruits (BOVY et al., 2007). However, some tomato wild species as *Solanum lycopersicoides*, *S. cheesmaniae* and *S. chilense* have certain anthocyanin levels in their fruits. The locus *Anthocyanin fruit* (*Aft*) was selected by the crossing between *S. lycopersicum* and *S. chilense*, this locus provides partial anthocyanin levels in epicarp fruits, being the synthesis largely regulated by light (MES et al., 2008). Furthermore, the recessive allele *atroviolacea* (*atv*), from *S. cheesmaniae*, provides the increase of the anthocyanin pigmentation mainly in the vegetative tissues when present in cultivated tomatoes. When the locus *Aft* is combined with *atv*, an anthocyanin increase is observed in the epicarp of tomato (MES et al., 2008). A triple mutant tomato (*Aft/atv/hp2*) with dark purple color in the fruit epicarp (SESTARI et al., 2014) was developed via Mendelian crossings. In addition to the anthocyanins, this triple mutant also showed high concentrations of ascorbate and lycopene, without any loss in yield.

For success in increasing anthocyanin accumulation in different genotypes, it is important to understand the genes related with the pathway and how they are regulated. The anthocyanin biosynthesis is one of the most well studied secondary metabolite pathway and are produced by a branch of the flavonoid pathway (RAHIM et al., 2014; WINKEL-SHIRLEY, 2001). Two gene types regulate the anthocyanin biosynthesis, the structural and regulatory genes (DOONER et al., 1991; SPRINGOB et al., 2003; KOES et al., 2005). The structural genes encode enzymes that catalyze each step of the pathway. They are divided in early biosynthetic genes (EBGs) that are common to different flavonoid sub-pathway, and the late biosynthetic genes (LBGs) which are related with the anthocyanin and proanthocyanidin (DUBOS et al., 2010; PELLETIER et al., 1999; NESI et al., 2000). These structural genes are regulated by the combined action of R2R3-MYB and the basic helix-loop-helix (bHLH) transcription factors (TFs), as well as WD40 proteins. These three transcription factors combine and form the ternary complex MBW, responsible to active the structural genes (DUBOS et al., 2010; STRACKE et al., 2007).

The microRNAs (miRNAs) are molecules of 20 to 24 nucleotides belonging to the small RNAs (sRNAs) class, having an important regulatory action in post-transcriptional processes in eukaryotic organisms (VOINNET, 2009). miRNAs are molecules that exhibit conserved behavior in the evolutionary question, thus demonstrating an evolutionarily conserved mechanism of gene regulation (MOLNÁR et al. 2007).

miRNA acts on the degradation or blocking of the target mRNA, regulating different processes, such as development, differentiation, metabolism and defense (VOINNET, 2009).

It is already known that miRNAs are responsible for important biological process, as leaf, flower, and fruit development, as well as biotic and abiotic stress response in tomato (KETAO et al., 2018; LI et al., 2017; LOPEZ-GOMOLLON et al., 2012; ORI et al., 2007; PAN et al., 2017). Several studies are being carried out to seek a better understanding of the regulatory mechanisms and processes of this type of molecule.

The participation of the miRNAs in the anthocyanin biosynthesis has already been reported in *Arabidopsis* (CUI et al., 2014; GOU et al. 2011; HSIEH et al. 2009; LEI et al., 2016; LUO et al. 2012; RAJAGOPALAN et al. 2006; YANG et al. 2013; ZHAO et al., 2017). However, in tomato the miRNAs role on this pathway is still not well characterized. A recent study showed that the miR858 targets the SlMYB7-like and SlMYB48-like genes. In the referred study, using a small tandem target mimic (STTM) to block the miR858, it was observed an up-regulation in the expression of these two anthocyanin genes, as well as the increase in the transcripts of several key anthocyanin biosynthesis pathway genes. Therefore, a purple phenotype with high anthocyanin accumulation was observed in leaf veins, stems and leaf buds (JIA et al., 2015).

In this context, it is possible to affirm that the miRNAs act in the anthocyanin biosynthesis pathway and a better understanding of their influence in tomato is necessary.

## 2. HYPOTHESIS

We hypothesize that the anthocyanin biosynthesis pathway in tomato (*Solanum lycopersicum*) is influenced by miRNAs action.

## 3. AIMS

### 3.1. General aim:

The aim of this work was the study of the miRNAs related with the anthocyanin biosynthesis pathway in tomato (*Solanum lycopersicum*).

### 3.2. Specific aims:

- Prediction of the miRNAs that putatively regulate the anthocyanin biosynthesis pathway genes in tomato.

- Validation of some miRNAs, that has anthocyanin biosynthesis pathway genes as target, by RT-qPCR in two tomato genotypes, Micro-Tom triple mutant (*Aft/atv/hp2*) and Micro-Tom Wild-Type.
- Knockout of the miR5368, miR6024 and miR9471 in Micro-Tom Wild-Type by CRISPR-Cas9.

## 4. REFERENCES

BORGHESI, E. et al. Effects of Salinity Stress on Carotenoids, Anthocyanins, and Color of Diverse Tomato Genotypes. **Journal of Agricultural and Food Chemistry,** v. 59, n. 21, p. 11676–11682, 2011.

BOVY, A.; SCHIJLEN, E.; HALL, R. Metabolic engineering of flavonoids in tomato (*Solanum lycopersicum*): the potential for metabolomics. **Metabolomics,** v. 3, n. 3, p. 399, 2007.

BUER, C. S.; IMIN, N.; DJORDJEVIC, M. A. Flavonoids: new roles for old molecules. **Journal of integrative plant biology,** v. 52, n. 1, p. 98-111, 2010.

BUTELLI, E. et al. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. **Nature biotechnology,** v. 26, n. 11, p. 1301-1308, 2008.

CHAREPALLI, V. et al. Anthocyanin-containing purple-fleshed potatoes suppress colon tumorigenesis via elimination of colon cancer stem cells. **The Journal of nutritional biochemistry,** v. 26, n. 12, p. 1641-1649, 2015.

CHRISTIE, P. J.; ALFENITO, M. R.; WALBOT, V. Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. **Planta,** v. 194, n. 4, p. 541-549, 1994.

CUI, L. et al. The miR156-SPL 9-DFR pathway coordinates the relationship between development and abiotic stress tolerance in plants. **The Plant Journal,** v. 80, n. 6, p. 1108-1117, 2014.

DIACONEASA, Z. et al. Antiproliferative and antioxidant properties of anthocyanin rich extracts from blueberry and blackcurrant juice. **International journal of molecular sciences,** v. 16, n. 2, p. 2352-2365, 2015.

DOONER, H. K.; ROBBINS, T. P.; JORGENSEN, R. A. Genetic and developmental control of anthocyanin biosynthesis. **Annual review of genetics,** v. 25, n. 1, p. 173-199, 1991.

DUBOS, C. et al. MYB transcription factors in Arabidopsis. **Trends in Plant Science,** v. 15, n. 10, p. 573–581, 2010.

FILGUEIRA, F. **Solanáceas:** Lavras: Editora UFLA, 2003.

GEORGIEVA, D. et al. Analytical features of an optimized method for HPLC analysis of some polyphenolic acids and flavonoids in tomato fruits. **Agricultural Science and Technology,** v. 6, n. 4, p. 480-485, 2014.

GOU, J. et al. Negative regulation of anthocyanin biosynthesis in Arabidopsis by a miR156-targeted SPL transcription factor. **The Plant Cell,** v. 23, n. 4, p. 1512-1522, 2011.

GU, L. et al. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. **Journal of Agricultural and Food Chemistry,** v. 51, n. 25, p. 7513-7521, 2003.

GUO, H.; LING, W. The update of anthocyanins on obesity and type 2 diabetes: experimental evidence and clinical perspectives. **Reviews in Endocrine and Metabolic Disorders,** v. 16, n. 1, p. 1-13, 2015.

HE, J. et al. Oxidative formation and structural characterization of new alpha-pyranone (lactone) compounds of non-oxonium nature originated from fruit anthocyanins. **Food Chemistry,** v. 127, n. 3, p. 984–992, 2011.

HSIEH, L. et al. Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. **Plant physiology,** v. 151, n. 4, p. 2120-2132, 2009.

JIA, X. et al. Small tandem target mimic-mediated blockage of microRNA858 induces anthocyanin accumulation in tomato. **Planta,** v. 242, n. 1, p. 283-293, 2015.

KETAO, W. A. N. G. et al. Identification and characterization of microRNA during Bemisia tabaci infestations in Solanum lycopersicum and Solanum habrochaites. **Horticultural Plant Journal,** v. 4, n. 2, p. 62-72, 2018.

KOES, R.; VERWEIJ, W.; QUATTROCCHIO, F. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. **Trends in plant science,** v. 10, n. 5, p. 236-242, 2005.

LEI, K. et al. Modulation of the phosphate-deficient responses by microRNA156 and its targeted SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 in Arabidopsis. **Plant and Cell Physiology,** v. 57, n. 1, p. 192-203, 2016.

LI, S. et al. Identification and characterization of Prunus persica miRNAs in response to UVB radiation in greenhouse through high-throughput sequencing. **BMC genomics,** v. 18, n. 1, p. 938, 2017.

LOPEZ-GOMOLLON, S. et al. Diverse correlation patterns between microRNAs and their targets during tomato fruit development indicates different modes of microRNA actions. **Planta,** v. 236, n. 6, p. 1875-1887, 2012.

LUO, Q. et al. An autoregulatory feedback loop involving PAP1 and TAS4 in response to sugars in Arabidopsis. **Plant molecular biology,** v. 80, n. 1, p. 117-129, 2012.

MES, P. J. et al. Characterization of tomatoes expressing anthocyanin in the fruit. **Journal of the American Society for Horticultural Science,** v. 133, n. 2, p. 262-269, 2008.

MOLNÁR, A. et al. miRNAs control gene expression in the single-cell alga Chlamydomonas reinhardtii. **Nature,** v. 447, n. 7148, p. 1126-1129, 2007.

NESI, N. et al. The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in Arabidopsis siliques. **The Plant Cell,** v. 12, n. 10, p. 1863-1878, 2000.

ORI, N. et al. Regulation of LANCEOLATE by miR319 is required for compound-leaf development in tomato. **Nature genetics,** v. 39, n. 6, p. 787-791, 2007.

PAN, C. et al. Identification and expression profiling of microRNAs involved in the stigma exsertion under high-temperature stress in tomato. **BMC genomics,** v. 18, n. 1, p. 843, 2017.

PELLETIER, M. K.; BURBULIS, I. E.; WINKEL-SHIRLEY, B. Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and end-products in Arabidopsis seedlings. **Plant molecular biology,** v. 40, n. 1, p. 45-54, 1999.

RAHIM, M. A.; BUSATTO, N.; TRAINOTTI, L. Regulation of anthocyanin biosynthesis in peach fruits. **Planta,** v. 240, n. 5, p. 913-929, 2014.

RAJAGOPALAN, R. et al. A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana. **Genes & development,** v. 20, n. 24, p. 3407-3425, 2006.

SARMA, A. D.; SHARMA, R. Anthocyanin-DNA copigmentation complex: mutual protection against oxidative damage. **Phytochemistry,** v. 52, n. 7, p. 1313-1318, 1999.

SESTARI, I. et al. Near-isogenic lines enhancing ascorbic acid, anthocyanin and carotenoid content in tomato (Solanum lycopersicum L. cv Micro-Tom) as a tool to produce nutrient-rich fruits. **Scientia Horticulturae,** v. 175, p. 111-120, 2014.

SPRINGOB, K. et al. Recent advances in the biosynthesis and accumulation of anthocyanins. **Natural product reports,** v. 20, n. 3, p. 288-303, 2003.

STRACKE, R. et al. Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. **The Plant Journal,** v. 50, n. 4, p. 660-677, 2007.

TANAKA, Y.; OHMIYA, A. Seeing is believing: engineering anthocyanin and carotenoid biosynthetic pathways. **Current opinion in biotechnology,** v. 19, n. 2, p. 190-197, 2008.

TOUFEKTSIAN, M. et al. Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. **The Journal of nutrition,** v. 138, n. 4, p. 747-752, 2008.

VOINNET, O. Origin, biogenesis, and activity of plant microRNAs. **Cell,** v. 136, n. 4, p. 669-687, 2009.

WINKEL-SHIRLEY, B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. **Plant physiology,** v. 126, n. 2, p. 485-493, 2001.

YANG, F. et al. Overexpression of microRNA828 reduces anthocyanin accumulation in Arabidopsis. **Plant Cell, Tissue and Organ Culture (PCTOC),** v. 115, n. 2, p. 159-167, 2013.

ZHAO, D. et al. Overexpression of herbaceous peony miR156e-3p improves anthocyanin accumulation in transgenic Arabidopsis thaliana lateral branches. **3 Biotech,** v. 7, n. 6, p. 379, 2017.

# PART 2: ARTICLE

# ARTICLE: microRNAs knockout induces anthocyanin accumulation in tomato

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# ABSTRACT

Anthocyanins are compound belonging to the flavonoid group and are related with important biological process in plants. In addition, anthocyanins have been related with prevention and protection against a range of chronic diseases, including different types of cancer. Tomato is the most vegetable consume in worldwide, however most tomato varieties do not accumulate anthocyanin in their fruits. By gene introgression from purple-fruit tomato wild species is possible to obtain fruits with purple pigmentation. Nonetheless, the high level of anthocyanin accumulation is restricted to the epidermal layers (peel). microRNAs belong to the group of non-coding endogenous RNAs and regulate gene expression post transcriptionally, by mRNA cleavage or suppressing translation. Here, by miRNA target prediction, we identified miR5368, miR6024-3p and miR9471b-3p as putative regulators of the genes anthocyanidin synthase (ANS), SlAN1 (bHLH), and chalcone synthase (CHS), respectively. The validation of these miRNAs was made by RT-qPCR in two tomato genotypes, Micro-Tom Wild-Type, which bears red-fruits, and Micro-Tom triple mutant (*Aft/atv/hp2*), which shows a purple phenotype in their fruits' epicarp. Furthermore, we also analyzed the expression of the anthocyanin biosynthesis genes ANS, CHS and SlAN1 by RT-qPCR. Our results revealed an inverse expression pattern between the miRNAs and its target genes in peel of two fruit developmental stages; green and physiologically mature. Aiming at better understand the miRNAs' roles on anthocyanin biosynthesis pathway, we performed the knockout of each miRNA precursor by CRISPR-Cas9 in the genotype Micro-Tom Wild-Type. As a result, the transformed explants showed a purple phenotype in the leaf veins and stem, indicating that the miR5368, miR6024 and miR9471 act on anthocyanin biosynthesis pathway by negatively regulating the expression of the genes ANS, SlAN1 and CHS respectively.

**Keywords:** Tomato; Anthocyanin Biosynthesis; microRNAs; CRISPR-Cas9.

# INTRODUCTION

Anthocyanins are compounds belonging to the flavonoid group, and represent a large group of plant secondary metabolites. The anthocyanins are related to important biological processes in plants, such as pollinators attraction and seeds dispersers as well as protection against biotic and abiotic stresses, i.e. high light intensity, cold temperatures, pathogens and injuries (Gould, 2004; Albert et al., 2009; Olsen et al., 2009; Zhang et al., 2013). In addition, the anthocyanins are responsible to the red, blue and purple pigmentation in different vegetables and fruits (Barrett et al., 2010; Jaakola, 2013), which it is possible to associate this different colors with benefits for human health (Pojer et al., 2013).

The anthocyanins also help to fight and prevent a range of chronic diseases. They have many proprieties, such as antioxidant effect, cardiovascular diseases prevention, cancer prevention, diabetes prevention, visual health improvement and neuroprotection (He et al., 2011; Khoo et al., 2017; Shim et al., 2012). However, it is important to highlight that these benefits are only achieved when a considerable amount of anthocyanins is regularly consumed (Butelli et al., 2008; Habanova et al., 2016). Unfortunately, the main vegetables present in our diet accumulate anthocyanin in small quantities in their edible parts. The high amount is restricted to the epidermal layers (peel/skin) (Butelli et al., 2008). Since the peel usually represents less than 5% of the total mass of edible parts of the plant (Sestari et al., 2014), a high anthocyanin accumulation in other edible parts of vegetables and fruits is desirable, so that we can benefit from these added characteristics.

Tomato is the most consumed and produced vegetable in the world (FAO, 2015). This high production and consumption can be explained by its flexibility, since it can be consumed fresh or in different ways processed by the industry (Filgueira, 2003). In addition, the tomato fruits have several nutrients essential for human health, as vitamins, lycopene, carotenoids and flavonoids (Georgieva et al., 2014). The fruit color is determined by the pigments quantity presented, for example, the lycopene is responsible for red coloring, the carotene for yellow coloring and the anthocyanin for the purple coloring (Filgueira, 2003).

Most tomato varieties do not accumulate anthocyanin in their fruits. However, it is possible to achieve this trait by genetic transformation as well as by gene introgression from purple-fruit wild species. The combination of the dominant allele *Anthocyanin fruit* (*Aft*) from *Solanum chilense* and the recessive allele *atroviolacea* (*atv*) from *S. cheesmaniae* into a cultivated tomato background leads to a purple-fruit with high anthocyanin amount in peel (Povero et al., 2011; Maligeppagol et al., 2013). Furthermore, the introgression of three

natural allelic variants from wild species (*Anthocyanin fruit* (*Aft*), *atroviolacium* (*atv*) and *high pigment 2* (*hp2*)), leaded to a genotype (*Aft/atv/hp2*) with dark purple color in the fruit epicarp (Sestari et al., 2014).

The anthocyanin biosynthesis is regulated by the regulatory and structural genes. The regulatory genes encode R2R3-MYB, basic helix-loop-helix (bHLH) and WD40-repeat (WDR) transcription factors. These three classes of transcription factors form a ternary complex known as MBW-complex, which bind to the promoter of the structural genes and regulate their expression (Bulgakov et al., 2017; Xu et al., 2015). Structural genes encode enzymes that act directly on the anthocyanin biosynthesis, each enzyme catalyze each reaction step (Dubos et al., 2010). They are divided in early biosynthetic genes (EBGs), that includes chalcone synthase (CHS), chalcone isomerase (CHI) and flavanone 3-hydroxylase (F3H); and late biosynthetic genes (LBGs), including flavonoid 3′-hydroxylase (F3′H) or flavonoid 3′,5′-hydroxylase (F3′5′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and flavonoid 3-O-glucosyltransferase (UFGT) (Liu et al., 2018). This regulation occurs differently in monocot and dicot plants. The structural genes in monocot plants are regulated by the MBW-complex, while in dicots, only the late biosynthetic genes require the MBW-complex (Petroni and Tonelli, 2011; Rahim et al. 2014; Gonzalez et al. 2008).

microRNAs (miRNAs) are a class of small non-coding RNAs that contain 21-24 nucleotides and regulate gene expression post transcriptionally, by mRNA cleavage or suppressing translation (Baulcombe 2004; Jones-Rhoades et al. 2006; Rhoades et al., 2002). It is already known that miRNAs are responsible for important biological process, as leaf, flower, and fruit development, as well as biotic and abiotic stress response in tomato (Ketao et al., 2018; Li et al., 2017; Lopez-Gomollon et al., 2012; Ori et al., 2007; Pan et al., 2017). However, there are only a few reports on the roles of miRNAs in anthocyanin biosynthesis pathway in tomato.

In *Arabidopsis*, it is already known that the miRNAs can influence, directly and indirectly, the anthocyanin biosynthesis pathway (Cui et al., 2014; Gou et al. 2011; Hsieh et al. 2009; Lei et al., 2016; Luo et al. 2012; Rajagopalan et al. 2006; Yang et al. 2013; Zhao et al., 2017). In tomato, the miRNAs role in this pathway is not very characterized yet. A study showed that the miR858 target the SlMYB7-like and SlMYB48-like genes. In the referred study, using a small tandem target mimic (STTM), to block the miR858, it was observed an up-regulation in the expression of these two anthocyanin genes, as well as the increase in the transcripts of several key anthocyanin biosynthesis pathway genes. Therefore, a purple

phenotype with high anthocyanin accumulation was observed in leaf veins, stems and leaf buds (Jia et al., 2015).

Based on this information, it is possible to affirm that the miRNAs act in the anthocyanin biosynthesis pathway in tomato and influence directly in the anthocyanin accumulation. Here, using a set of methodologies, as miRNA target prediction, RT-qPCR and CRISPR-Cas9, we observed that the knockout of the miR5368, miR6024 and miR9471 here predicted to target the genes ANS, SlAN1 and CHS, respectively, in tomato lead to purple phenotype in leaf veins and stem. This is the first study that validates the expression of these novel miRNAs by RT-qPCR in tomato, and identified them as influencer in anthocyanin biosynthesis pathway.

# MATERIAL AND METHODS

## miRNA target prediction

Initially it was made a selection, based on literature, of the main genes related with anthocyanin biosynthesis pathway in tomato. For the prediction analysis of possible miRNAs acting on these genes, it was used the *S. lycopersicum* miRNAs identified by Cardoso et al. (2018). The miRNA targets were predicted using the psRNATarget tool (http://plantgrn.noble.org/psRNATarget/) (Dai; Zhuang; Zhao, 2018), using a strict threshold, being 3.0 for "Maximum expectation (Exp)". Other parameters were as default.

## Plant material

The tomato (*Solanum lycopersicum* L) cultivar Micro-Tom (MT) (Meissner et al., 1997) and Micro-Tom triple mutant (*Aft/atv/hp2*) (Sestari et al., 2014) were used. Plants were grown under 16 h light/ 8 h dark cycle at 25 °C. Mature leaves were collected and fruits in two ripening stages, green and physiologically mature, had peel and flesh dissected. The material was collected from three individual plants and together they represented one biological replication. Three biological repetitions were used in the analyses. All plant material was immediately frozen in liquid nitrogen and stored in a −80 °C freezer until RNA extraction.

## RNA extraction and treatment with DNase

Total RNA was extracted by Trizol reagent (Invitrogen). The integrity of the RNA was visualized in 0.8% agarose gel, and the quantity and quality (ratio 260/280 and 260/230 between 1.8 and 2.2) were measured on Nanovue spectrophotometer. RNA samples (5.0µg) were treated with Turbo DNA-free™ kit (Life Technologies™) for elimination of residual DNA and the samples were stored at −20 °C until cDNA synthesis.

## cDNA synthesis

For the cDNA synthesis and miRNA expression the stem-loop method (Chen et al., 2004; Varkonyi-Gasic et al. 2007) was used. This method requires the use of three different primers: the stem-loop RT primer, the forward primer and the reverse universal primer. The stem-loop primers were designed according to Chen (2004). The stem-loop RT primer is formed by 50 nucleotides, of which the first 44 nucleotides correspond to a universal sequence that forms a stable stem-loop structure at low temperatures. This primer contains the last 6 nucleotides, at the 3' end, complementary to the last 6 nucleotides present at the 3' end of the specific mature miRNA. The forward primers contain the exact sequence of the mature miRNA, excluding the last 6 nucleotides at the 3' end. Aiming to improve the melting temperature, 5-7 nucleotides were randomly added at the 5' end of the forward primers. The OligoAnalyzer tool from Integrated DNA Technologies website (https://www.idtdna.com/calc/analyzer) was used to verify the quality of the primers as well as to calculate the melting temperature. The reverse primer is the reverse complement of the universal sequence present in the stem-loop RT primer and it can be used for every miRNA. Primers forward and reverse for anthocyanin genes and reference genes were designed based on the gene sequences available in the Sol Genomic Networks (https://solgenomics.net/). All primers sequences are listed in Supplementary table 1.

For the cDNA synthesis, the *ImProm-II^Tm^ Reverse Transcriptase* (Promega) kit was used with the total (DNA-free) RNA, according with the following steps. For each miRNA reaction, 500 ng of RNA was used. After the calculation according with the RNA concentration, the volume was adjusted to 7 µL with nuclease-free water and 1 µL of oligo-dT primer, 2 µL of specific stem-loop RT primer (1 µM) and 1 µL of the dNTP mix were added. The samples were incubated at 70 °C for 10 minutes for denaturation of the secondary structures and later incubated at 4 °C for 10 minutes. Then, 5 µL of Improm-II 5 × reaction

buffer, 2.4 µL of $MgCl2$ (25mM), 0.6 µL of RNaseOut (Invitrogen) and 1 µL of the Improm-II Reverse Transcriptase were added. These final reactions were incubated in a thermocycler at 16 °C for 30 minutes, followed by reverse transcription of 60 cycles at 30 °C for 30 seconds, 42 °C for 30 seconds and 50 °C for 1 second. At the end the reactions was incubated at 70 °C for 15 minutes to inactivation of the Improm-II Reverse Transcriptase. The cDNA were stored at −20 °C. Because we used the oligo-dT primer we were able to analyze the expression of the anthocyanin genes as well as the reference genes in this same cDNA method.

### Quantitative RT-qPCR

For the quantitative real-time PCR (RT-qPCR) the standard QuantiNova SYBR® Green PCR Kit (QIAGEN) protocol was used. The compounds of these reactions were 1.5 µL cDNA, 1.5 µL of each primer (forward and reverse universal) at a final concentration of 1 µM, 7.5 µL of QuantiNova SYBR Green PCR Master Mix and 3.0 µL of water to a final reaction volume of 15 µL, for each reaction. These final reactions were incubated at 95 °C for 2 minutes, followed by 40 cycles at 95 °C for 5 seconds and 60 °C for 10 seconds. Then, the samples were heated from 55 to 95 °C with an increase of 1 °C to acquire the melting curve of the amplified products. All reactions were run in duplicate.

The primer efficiency was calculated for each miRNA, anthocyanin gene and the reference gene by a standard curve of a 1:5 serial dilution of a cDNA pool. For the calculation of relative expression, the normalized comparative Cq (quantitative Cycle) method was used (Pfaffl, 2001), using *Solanum* U6 and 5.8S as reference genes.

### Statistical analyses

Statistical analyses were performed by one-way ANOVA followed by the Student's *t*-test to establish significant differences between means $p$ value < 0.05.

### Guide RNA design, CRISPR-Cas9 vectors and plant transformation

The CRISPR-Cas9 vectors were designed using the protocol 3A of Čermák et al. (2017). The vectors were made by the direct assembly of gRNAs into the T-DNA vector pDIRECT_22C. The highest deletion efficiency is achieved with the use of two gRNAs (Čermák et al. 2017). Therefore, two gRNAs were used to induce the knockout of one

miRNA. Since the mature miRNA is a small sequence, it is not be possible to design two gRNAs to effectively delete only its sequence. Thus, we designed the gRNAs to target the region of the miRNA precursor (pre-miRNA). The mature and precursor miRNAs sequences and their position in the genome are available in Supplementary Table 2.

We constructed four vectors: pDIRECT_22C_miR5368; pDIRECT_22C_miR6024; pDIRECT_22C_miR9471b, and pDIRECT_22C_miR9471 targeting the knockout of the miR5368; miR6024; miR9471b; and miR9471a and miR9471b, respectively. For the CHS gene, our miRNA target prediction found that the miR9471b-3p is the possible regulator. The miR9471b-3p mature sequence is very similar to the miR9471a-3p, differing only by one nucleotide (Supplementary Table 2). So, besides the vector pDIRECT_22C_miR9471b, we decided to construct the vector pDIRECT_22C_miR9471 that can leads to the knockout of miR9471a and miR9471b concomitantly.

The gRNAs were designed using the software CHOPCHOP (https://chopchop.cbu.uib.no/). The miR5368 precursor is located at the intron of the gene Solyc08g061160, thus, the gRNA1 and gRNA2 in the vector pDIRECT_22C_miR5368 were designed to target this region and generate a deletion of 471 bp. The miR6024 precursor is located at the intergenic region close to the gene Solyc01g088430, thus, the gRNA1 and gRNA2 of the vector pDIRECT_22C_miR6024 were designed to target this region and generate a deletion of 170 bp. The miR9471b and miR9471a are located at the intron of the gene Solyc12g008590, thus, the gRNA1 and gRNA2 of the vector pDIRECT_22C_miR9471b as well as the vector pDIRECT_22C_miR9471 were designed to target this region and generate a deletion of 483 bp and 1749 bp respectively. All gRNAs were designed based on tomato genome SL2.50 version obtained from Sol Genomics Network (https://solgenomics.net/). The gRNAs sequences are available in Supplementary Table 3.

To produce the vector, initially, specific primers (Supplementary Table 4) were used to generate the gRNAs fragments by PCR reaction. These fragments were introduced in one step into pDIRECT_22C by Golden Gate assembly method described by Čermák et al. (2017). The cauliflower mosaic virus (CaMV) 35S promoter was used to drive the expression of Csy4 fused with *Arabidopsis* Cas9 (AtCas9). Another cauliflower mosaic virus (CaMV) 35S promoter was used to drive the Kanamycin resistance gene expression. The gRNAs array is regulated by the *Cestrum yellow leaf curling virus* (CmYLCV) promoter and, in the final array, each gRNA was separated by one Csy4-biding site.

Each vector was introduced into *E. coli* (NEB 10-beta Competent *E. coli*) by heat shock, accordingly to the manufacturer's protocol. Then, transformed *E. coli* were selected on

LB medium plates containing the antibiotic kanamycin (50 mg $L^{-1}$). One colony was selected and sequenced to confirm the correct assembly, for each vector. After the confirmation, the plasmid DNA was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The electroporation apparatus (Bio-Rad Nanopulser) was set to 2.5 kV, 25 µF capacitance, 400 Ω resistance. 30 µL of electro-competent cells and 5 µl of plasmid DNA (150 ng/µl) were added together in a cuvette and placed in the apparatus. Immediately after the electroporation, 300 µL YM medium was added to the cuvette and mixed carefully. The cuvette content was transferred to a culture tube and incubated at 28 °C for 4 hours at 250 RPM. Then, 200 µL of this *Agrobacterium* cell suspension was spread in a plate containing solid YM medium with 50 mg $L^{-1}$ kanamycin, 20 mg $L^{-1}$ rifampicin, and 100 mg $L^{-1}$ streptomycin, and incubated at 28 °C. The transformation was confirmed by colony PCR.

The tomato (*Solanum lycopersicum* L) cultivar Micro-Tom (MT) transformation was performed as described by Pino et al. (2010).

# RESULTS

## miRNA target prediction

The main known anthocyanin biosynthesis related genes in tomato were selected for miRNA target prediction, including the regulatory and structural genes (Supplementary table 5). It was predicted that some genes could be regulated by just one miRNA or more than one miRNA. We selected some of them to validate their expression by RT-qPCR (Table 1).

| Gene | Solyc ID | miRNA |
|---|---|---|
| SlJAF13 | Solyc08g081140 | sly-miR7696c-3p |
| SlAN1 | Solyc09g065100 | sly-miR6024-3p / sly-miR157a-1-3p |
| Anthocyanidin synthase (ANS) | Solyc08g080040 | sly-miR5368 |
| Chalcone Synthase (CHS) | Solyc09g091510 | sly-miR9471b-3p / sly-miR168a-2-3p |

**Table 1.** Anthocyanin related genes and their possible miRNAs regulators. The first column contain the anthocyanin related gene; the second their Solyc ID; and the third the possible miRNAs regulators of each gene.

It is already known that miR157 and miR168 are responsible for important biological processes in plants. In tomato, the miR157 target the transcription factor *Squamosa promoter-binding protein* (SBP)/*Squamosa promoter binding-like protein* (SPL) (Zhang et al., 2008, Yin et al., 2008, Luan et al., 2014). This group plays significant roles during the entire process of flowering in tomato (Salinas et al., 2012). In *Arabidopsis* the miR168 is responsible to

controls AGO1 homeostasis during ABA treatment and abiotic stress responses (Li et al., 2012). Therefore, we decided not to use these two miRNA in our subsequent analyses because the knockout of these miRNAs in tomato could lead to negative effects in these important biological processes.

## Expression analyses of miRNAs and their target genes

To validate the miRNAs identified in our target prediction and correlate the expression with the anthocyanin genes, RT-qPCR was used. The validation was made in fruits in two ripening stages, green and physiologically mature, from the two genotypes, *Solanum lycopersicum* cultivar Micro-Tom Wild Type (WT) and *Solanum lycopersicum* cultivar Micro-Tom Triple Mutant (*Aft/atv/hp2*).

### miR5368 and ANS gene

The miR5368 showed high expression level in all samples analyzed in Micro-Tom *Aft/atv/hp2* as well as in Micro-Tom WT, their amplification cycle was very early, between 19 and 20 in peel and flesh, in both genotypes analyzed. In Micro-Tom *Aft/atv/hp2* the highest expression was observed in peel compared to the flesh in both fruit development stages (Figure 1a), whereas, in Micro-Tom WT the highest expression was observed in flesh compared with peel in both fruit development stages (Figure 1b). There was no significant difference between the expression of these miRNAs in peel and flesh of each development stages in each genotype analyzed. In leaves, the expression of this miRNAs was also high in both genotypes (Supplementary material 1).

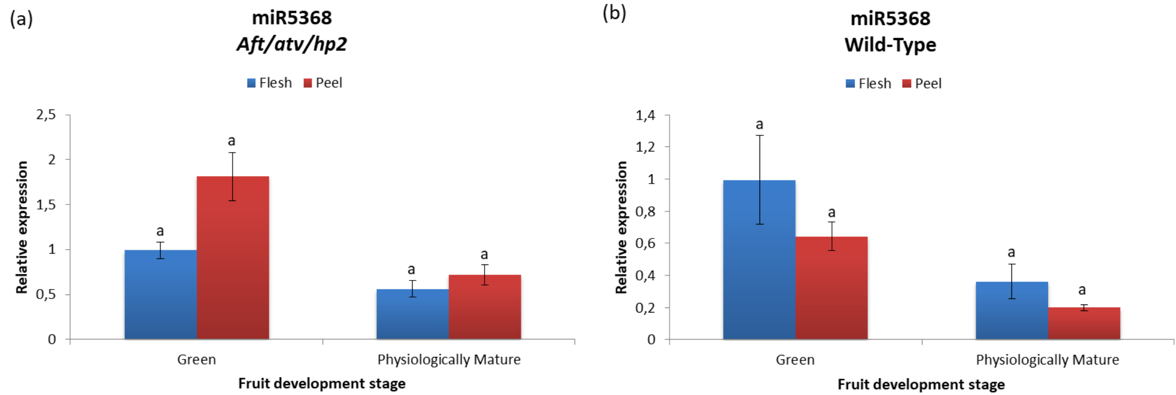



**Figure 1.** Relative expression pattern of miR5368 in peel and flesh of Micro-Tom *Aft/atv/hp2* (a) and Micro-Tom Wild-Type (b). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.

The ANS expression in the triple mutant *Aft/atv/hp2* was significant higher in peel than in flesh in both development stages (Figure 2a). This expression pattern was expected since the peel shows strong purple pigmentation compared with flesh. Also, in this genotype the expression of this gene in peel was up-regulated during the fruit ripening, showing expression of 3-fold higher in physiologically mature fruit than the green fruit (Figure 2a). The quantitative expression analysis of this anthocyanin gene in Micro-Tom WT showed a distinct expression pattern from that observed in Micro-Tom *Aft/atv/hp2*. The higher expression of ANS was in flesh of green fruits, while the other tissues showed lower expression. The expression between peel and flesh was significantly different only at green stage (Figure 2b).

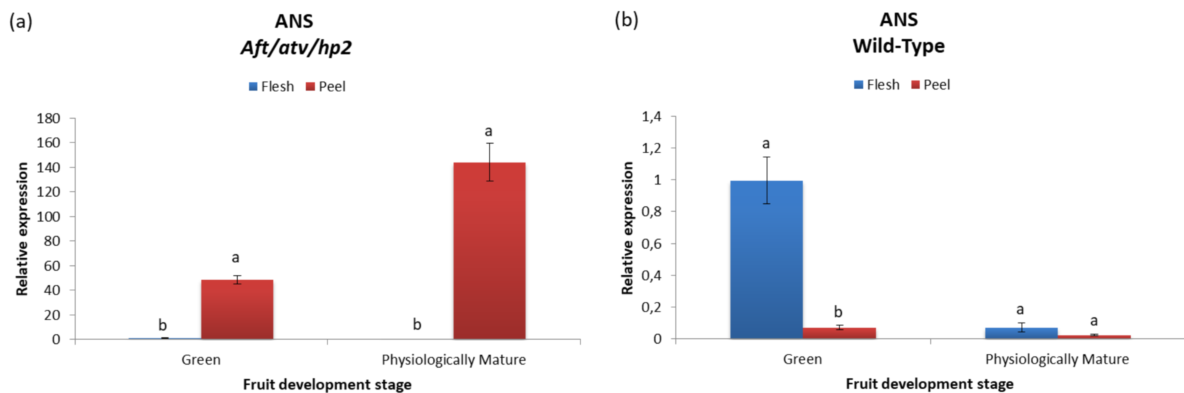



**Figure 2.** Relative expression pattern of ANS in peel and flesh of Micro-Tom *Aft/atv/hp2* (a) and Micro-Tom Wild-Type (b). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.

## miR6024-3p and SlAN1 gene

The miR6024-3p also showed high expression level in all samples, showing amplification cycles between 19 and 20 in peel and flesh, in both genotypes analyzed. In Micro-Tom *Aft/atv/hp2* the high expression was observed in peel compared with flesh in both fruit development stage (Figure 3a). In Micro-Tom WT at green stage it was observed a high expression in peel than in flesh, whereas, at physiologically mature stage it was observed a high expression in flesh than in peel (Figure 3b). There was significant difference in the expression of this miRNA, between peel and flesh, in the physiologically mature stage of *Aft/atv/hp2* and the green stage of WT. In leaves, the expression of miR6024-3p was also high in both genotypes (Supplementary material 1).

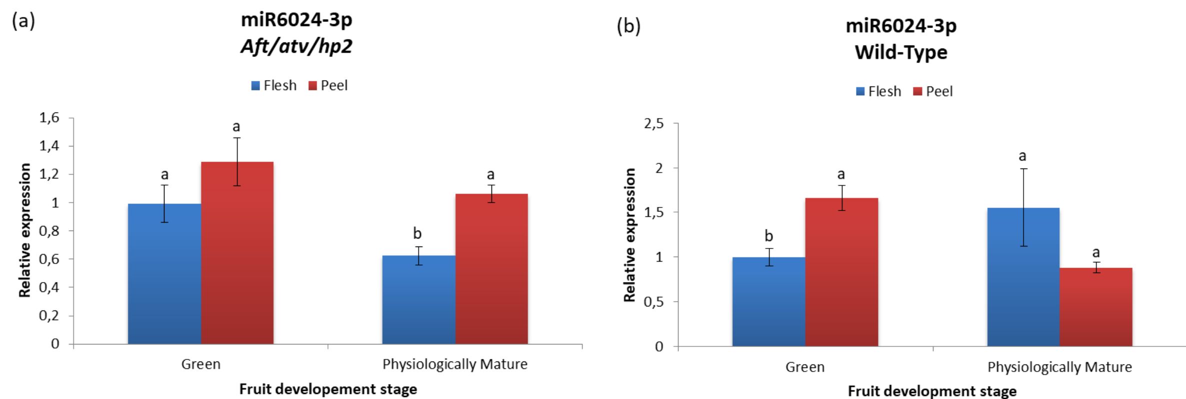



**Figure 3.** Relative expression pattern of miR6024-3p in peel and flesh of Micro-Tom *Aft/atv/hp2* (a) and Micro-Tom Wild-Type (b). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.

In Micro-Tom *Aft/atv/hp2* the SlAN1 expression was higher in peel than in flesh in both development stages, being significantly different (Figure 4a). Also in this genotype, the expression of this gene in peel was up-regulated during the fruit ripening, showing expression of 6.0 times higher in physiologically mature stage than in green stage (Figure 4a). The SlAN1 expression in Micro-Tom WT was very low for all samples, indicating just a few SlAN1 transcripts in this genotype. The high expression was observed in flesh of green fruits, while the other tissues showed lower expression. The expression between peel and flesh was significantly different only in the physiologically mature stage (Figure 4b).

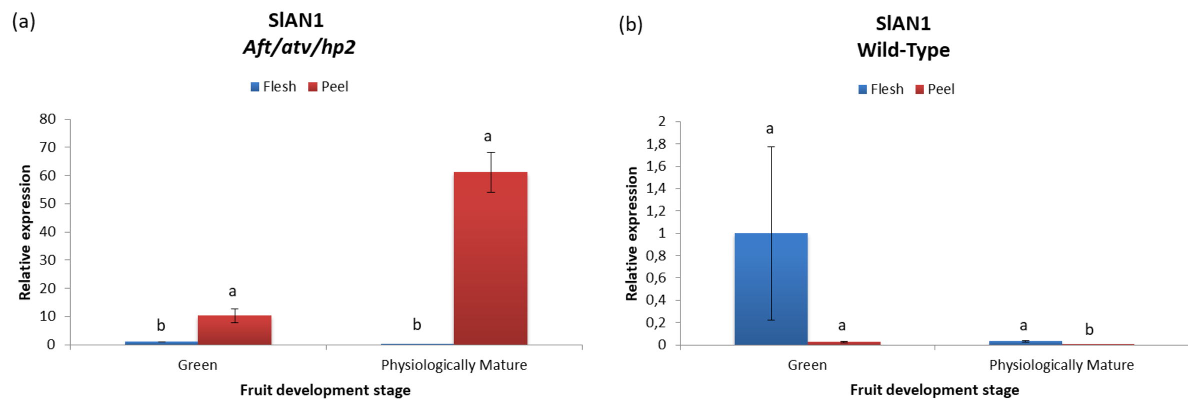



**Figure 4.** Relative expression pattern of SlAN1 in peel and flesh of Micro-Tom *Aft/atv/hp2* (a) and Micro-Tom Wild-Type (b). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.

## miR9471b-3p and CHS gene

The miR9471b-3p, as well as the miR5368 and miR6024-3p, showed high expression level in all samples analyzed in both genotypes. Its expression level was even higher than the other miRNAs, showing amplification cycle between 15 and 16 for all tissues and development stages in both genotypes analyzed. In Micro-Tom *Aft/atv/hp2* at green stage, the high expression of this miRNA was observed in flesh than in peel, whereas, at physiologically mature stage the high expression was observed in peel compared with flesh (Figure 5a). In Micro-Tom WT it was observed higher expression in flesh than in peel for both development stages (Figure 5b). In leaves, the expression of miR9471b-3p was also high in both genotypes (Supplementary material 1).

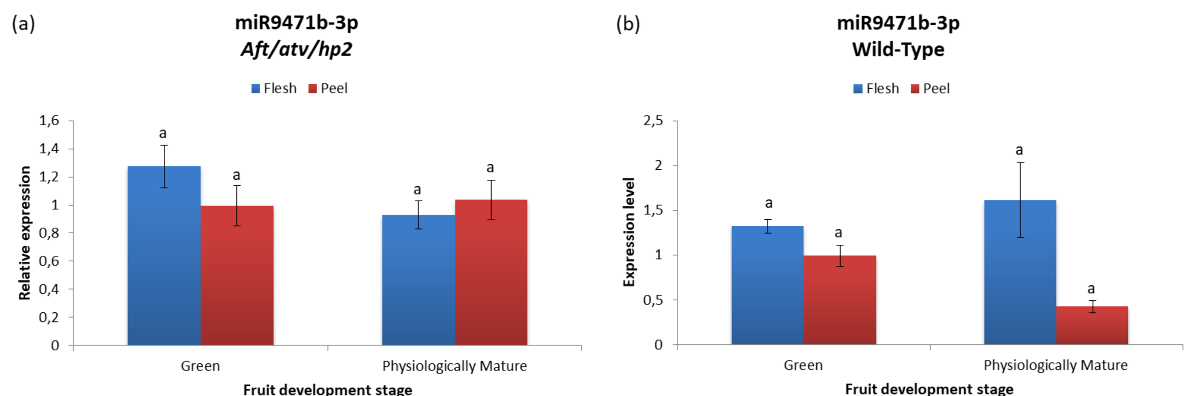



**Figure 5.** Relative expression pattern of and miR9471b-3p in peel and flesh of Micro-Tom *Aft/atv/hp2* (a) and Micro-Tom Wild-Type (b). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.

In Micro-Tom *Aft/atv/hp2* the expression of CHS was higher in peel than in flesh in both development stages, being significantly different (Figure 6a). Also in this genotype, the expression of this genes in peel were up-regulated during the fruit ripening, showing expression of 3.5 times higher in the physiologically mature stage than in green stage (Figure 6a). In Micro-Tom WT, the CHS expression was higher in peel of the physiologically mature stage, whereas, at the green stage there was no significant difference between the expressions in peel and flesh (Figure 6b).

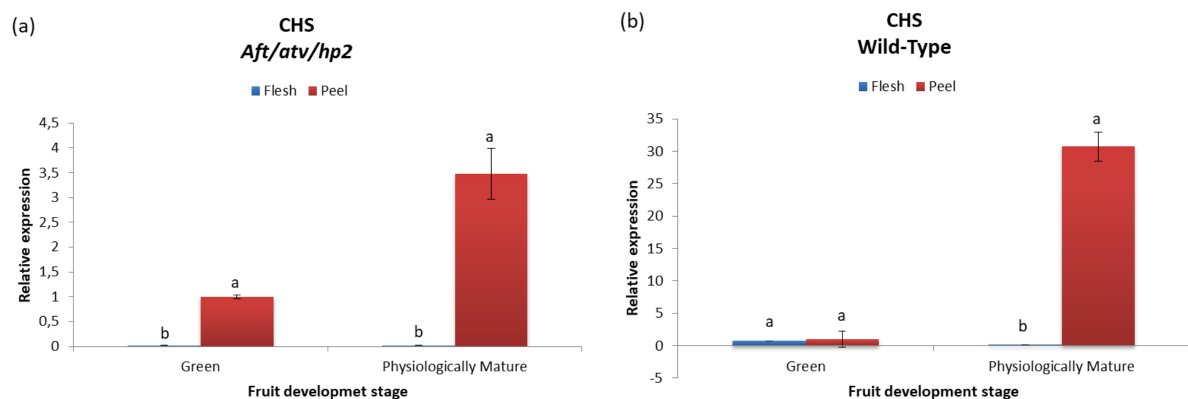



**Figure 6.** Relative expression pattern of and CHS in peel and flesh of Micro-Tom *Aft/atv/hp2* (a) and Micro-Tom Wild-Type (b). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.

## miR7696c-3p and SlJAF13

For miR7696c-3p, we did not detect any expression in the samples analyzed. Differently from the others anthocyanin genes analyzed, the SlJAF13 did not show increase in its expression from the green stage to the physiologically mature stage in Micro-Tom *Aft/atv/hp2*. The higher expression in this genotype was observed in peel of the green stage, and this expression was significantly different from flesh at this fruit development stage (Figure 7a). In Micro-Tom WT, the higher expression of SlJAF13 was observed in peel of green fruits and there was no significant difference in its expression between peel and flesh in both fruit development stage (Figure 7b).

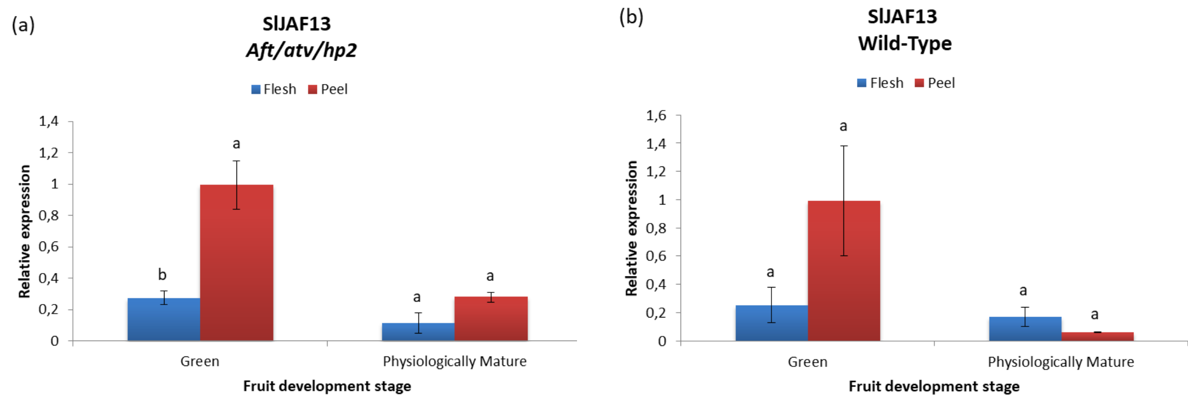



**Figure 7.** Relative expression pattern of SlJAF13 in peel and flesh of Micro-Tom *Aft/atv/hp2* (a) and Micro-Tom Wild-Type (b). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.

Comparing the expression of each miRNA and each anthocyanin gene analyzed, we observed an inverse expression level in peel between the green stage and physiologically mature stage from Micro-Tom *Aft/atv/hp2*. The expression of miR5368 and miR6024-3p was higher at the green stage compared with the physiologically mature stage (Figure 1a and 3a). For miR9471b-3p, the expression was almost the same in both stages (Figure 5a). Whereas, the expression of the anthocyanin genes ANS, SlAN1 and CHS was lower at the green stage and higher at physiologically mature stage (Figure 2a / 4a / 6a).

## Knockout of MIR gene

The miR5368, miR6024-3p and miR9471b-3p showed high expression levels in both genotypes indicating that they could strongly influence in their target genes expression, so we decided to proceed with the knockout of each miRNA precursor in Micro-Tom WT. The transformation process was made as described by Pino et al. (2010). In all the transformed explants with the knockout of each miRNA precursor (miR5368, miR6024, miR9471 and miR9471b) we observed a purplish phenotype in the leaf veins of the regenerant plantlets *in vitro* while the control remained green (Figures 8 – 12).

The explants containing the vectors pDIRECT_22C_miR9471 and pDIRECT_22C_miR9471b that promotes the knockout of miR9471a / miR9471b and just miR9471b respectively, predicted to control the expression of CHS, were the ones that showed a better response in anthocyanin accumulation. In these transformed explants, it was possible to see a purple phenotype in stems as well as in the leaf veins (Figures 8 and 9).

**Figure 8.** Micro-Tom Wild-Type explants transformed with the vector pDIRECT_22C_miR9471 that promotes the miR9471a and miR9471b knockout, predicted to control the expression of the CHS (Solyc09g091510) gene. Phenotype of 45-day-old transformed explants.

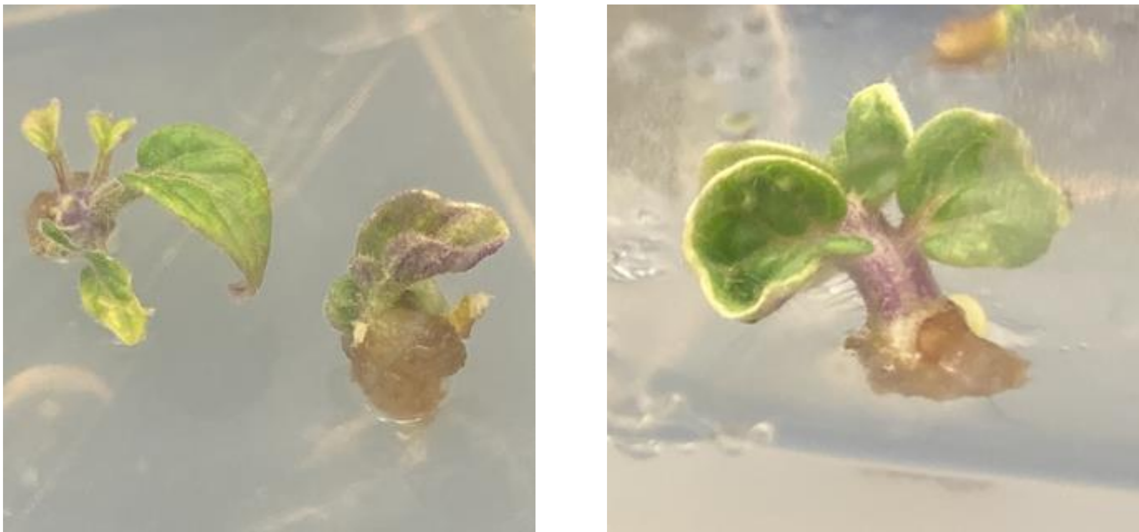


**Figure 9.** Micro-Tom Wild-Type explants transformed with the vector pDIRECT_22C_miR9471b that promotes the miR9471b knockout, responsible to control the expression of the CHS (Solyc09g091510) gene. Phenotype of 45-day-old transformed explants.

The explants containing the vector pDIRECT_22C_miR6024, that promotes the knockout of miR6024, predicted to control the expression of SlAN1, showed a purple phenotype in the leaf veins (Figure 10).

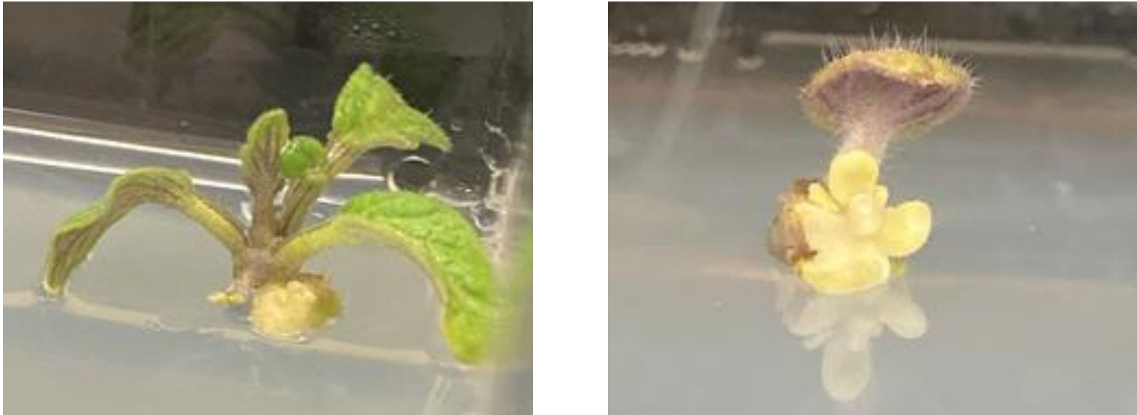


**Figure 10.** Micro-Tom Wild-Type explants transformed with the vector pDIRECT_22C_miR6024 that promotes the miR6024 knockout, predicted to control the expression of the SlAN1 (Solyc09g065100) gene. Phenotype of 45-day-old transformed explants.

The explants containing the vector pDIRECT_22C_miR5368 that promotes the knockout of the miR5368, predicted to control the expression of ANS, showed purple phenotypes in specific points of the transformed explants (Figure 9).

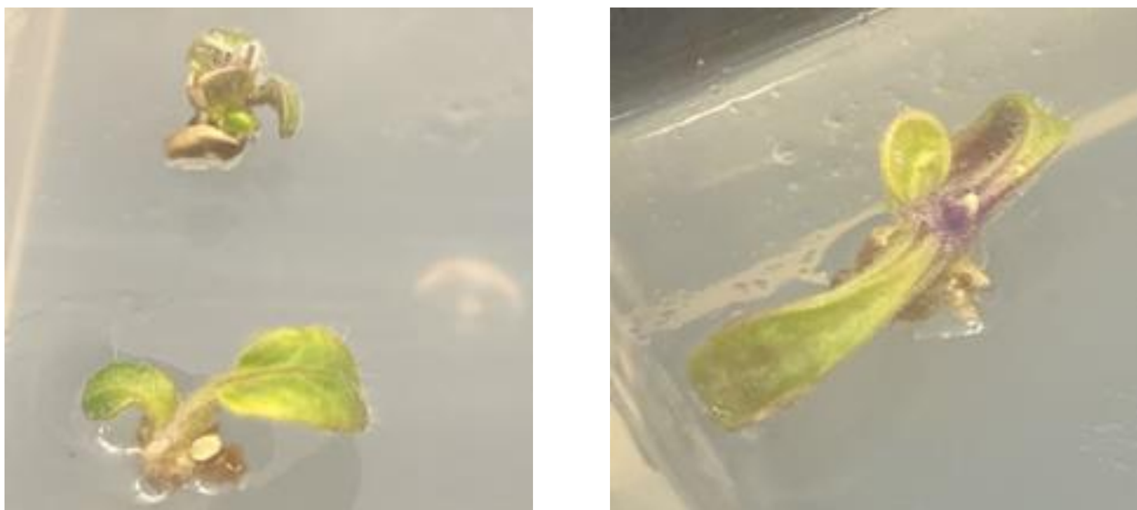


**Figure 11.** Micro-Tom Wild-Type explants transformed with the vector pDIRECT_22C_miR5368 that promotes the miR5368 knockout, predicted to control the expression of ANS (Solyc08g080040) gene. Phenotype of 45-day-old transformed explants.

**Figure 12.** Micro-Tom Wild-Type non-transformed explants (control).

# DISCUSSION

Aiming for a better understanding of the influence of the miRNAs in the anthocyanin biosynthesis pathway in tomato, we performed the prediction, validation and knockout of some miRNAs as well as the expression analyzes for some of the anthocyanin pathway related genes.

It is the first time that the miR5368, miR6024-3p, miR9471b-3p and miR7696c-3p are predicted to act in the anthocyanin biosynthesis pathway, more specifically in ANS, SlAN1, CHS and SlJAF13 genes, respectively. As novel miRNAs recently discovered, their biological functions remain unknown until now.

**SlJAF13 have constitutive expression.** In *Solanaceae*, the SlJAF13 and SlAN1 are the two main bHLH clades involved in anthocyanin biosynthesis (Spelt et al., 2000). The bHLH transcription factors, along with MYB and WD40 factors combine and form the ternary complex MBW, responsible to active structural genes expression in anthocyanin biosynthesis pathway (Dubos et al., 2010; Stracke et al., 2007). The bHLH in the ternary complex is responsible to recognize the transcription factor biding site in the target gene promoter (Montefiori et al., 2015).

The expression pattern of SlJAF13 was similar in both genotypes analyzed (Figure 7), which are in agreement with a previous work in tomato, where the SlJAF13 did not differ from the top and bottom of fruits from *Aft* and WT genotypes (Colanero et al., 2019). This similar expression between anthocyanin-pigmented and non-pigmented tomato confirm that SlJAF13 has a constitutive expression.

Although the miR7696c-3p was predicted to be present in tomato genome (Cardoso et al., 2018), the RT-qPCR essay did not detect any expression in the tissues and conditions tested. This miRNA was predicted to target the SlJAF13 gene, but further investigation is needed.

**The miR6024 knockout leads to purple phenotype in leaf veins of transformed explants.** The SlAN1 gene, together with the MYB and WD40 factors form the second MBW ternary complex responsible to up-regulate the expression of the LBGs (Liu et al., 2018). The RT-qPCR analyzes showed that the SlAN1 was higher expressed in peel of Micro-Tom *Aft/atv/hp2* compared with flesh in both fruit development stages (Figure 4a). This high expression of SlAN1 in peel was expected and is in agreement with other studies that analyzed this same gene in anthocyanin-pigmented tomatoes (Cao et al., 2017; Colanero et al., 2019; Qiu et al., 2016; Spelt et al., 2000), since this gene shows an important function in the composition of the MBW complex for the activation of the subsequent anthocyanin pathway genes. This indicates that the anthocyanin biosynthesis is inhibited in some way in the tomato flesh, being the miRNAs a possible negative regulator in this tissue.

The miR6024 was previously correlated with the cleavage of different disease resistance genes in plants. Initially, the miR6024 was identified to regulate the expression of *Tm-2* in tomato (Li et al., 2012). Furthermore, the homologues of the typical NB–LRR gene I2 in tomato were shown to be targeted by miR6024 instead of by miR482 (Wei et al., 2014). A study using tomato in two different conditions, infected with yellow leaf curl Sardinia virus (TYLCSV) compared to the virus-free plant, identified miR6024 as targeting the mRNA transcript RX-coiled-coil (RX-CC), Nucleotide Binding Site (NBS) and Leucine-Rich (LRR), which transcripts were down-regulated and cleaved only in the virus-infected tissues (Chiumenti et al., 2018). It is already known that one miRNA is capable to act on different targets (Guan et al. 2014; Xia et al. 2012). According to our target prediction, the miR6024-3p could regulate the SlAN1 gene expression. The expression of this miRNA was validated in all tissues and development stage in both genotype analyzed (Figure 3).

The miR6024-3p expression, in the triple mutant *Aft/atv/hp2*, was higher at the green stage than at the physiologically mature stage and the SlAN1 expression was higher in physiologically mature stage than in green stage. The same opposite correlation in the expression pattern between miRNA and target gene observed in previously work with miR858 and the anthocyanin related genes SlMYB7-like and SlMYB48-like (Jia et al., 2015). To further investigate the correlation between the expression of the miR6024-3p and SlAN1,

in the two genotypes of Micro-Tom, we decided to validate our miRNA target prediction analyzes by CRISPR-Cas9 in Micro-Tom WT. The transformed explants with the vector pDIRECT_22C_miR6024, designed to knockout the miR6024 precursor, showed a purple phenotype in leaf veins (Figure 10). The knockout of this miRNA probably leaded to an increase of the SlAN1 transcripts and consequently more MBW complex was formed being possible to activate the LBGs, leading to a high anthocyanin production in the leaf veins.

**The miR5368 knockout leads to purple phenotype in specific points of the knockout explants.** The ANS gene is one of the late biosynthesis genes (LBGs), and it is involved in the biosynthesis of several flavonoids, including anthocyanins. In tomato, as well as in many others *Solanaceae* vegetables, the expression levels of the LBGs is positively related with anthocyanin accumulation (André et al., 2009; Aza-Gonzalez et al., 2013; Borovsky et al., 2004; Povero et al., 2011). It was observed that the expression of the ANS was higher in the anthocyanin-pigmented mutants (*Aft/Aft*, *atv/atv,* and *Aft/Aft atv/atv*) compared to their red-fruited controls (Povero et al., 2011; Sapir et al., 2008). Similarly, we observed a higher ANS expression in peel of the triple mutant *Aft/atv/hp2* compared with WT genotype, being significantly different in both development stages (Supplementary material 2). In the triple mutant *Aft/atv/hp2*, the expression of ANS was higher in peel compared with flesh (Figure 2a), the same expression pattern observed by Kang et al. (2018) in the genotype SAM containing purple fruits. This expression pattern was expected, since the flesh does not accumulate anthocyanin.

miR5368 was first identified by Solexa sequencing in libraries from water deficit and rust infection in *Glycine max*. In the referred study, miR5368 was predicted to target the glucuronosyl/glucosyl transferase and GTPase-activating protein genes (Kulcheski et al., 2011), but validation of expression and target was not performed. In alfalfa, the miR5368 was identified as a drought-responsive miRNA, being down-regulated in stressed leaves (Li et al., 2017), but the target of this miRNA remained unknown. In tomato, the miR5368 was identified *in silico*, by Cardoso et al. (2018). Here, we predicted ANS as a target of miR5368. The validation showed high expression level in all tissues and development stages analyzed in both genotype, Micro-Tom *Aft/atv/hp2* and Micro-Tom WT (Figure 1).

The expression pattern for this miRNA and his target gene, in the peel of both fruit development stages, also showed the same opposite correlation expression pattern for miRNA and target gene observed here for miR6024 and SlAN1.

After obtaining this information and correlation about the expression of the miR5368 and ANS, in both genotypes, we proceed with the validation of this miRNA target prediction analyzes by CRISPR-Cas9 in Micro-Tom WT.

The transformed explants with the vector pDIRECT_22C_miR5368, designed to knockout the miR5368 precursor, showed points with purplish phenotype (Figure 11). Compared with the other results found for the other miRNA, the miR5368 precursor knockout plant seems not to be so efficient in anthocyanin accumulation. This happens probably because the increase of just ANS is not sufficient to promote the anthocyanin accumulation, since it is present almost at the end of the pathway. Probably it did not have enough substrate to act on and promote a high anthocyanin production.

**The miR9471 knockout leads to purple phenotype in leaf veins and stems.** The CHS gene is one of the EBGs, and is involved in the biosynthesis of all flavonoids. The anthocyanins are one of the secondary metabolites as well as the flavonoids, condensed tannins and isoflavonoids (Williams and Grayer, 2004). In *Solanaceae*, the expression pattern of the EBGs varies, therefore not being possible to draw a consistent correlation between their expression levels and the anthocyanin accumulation (Aza-Gonzalez et al. 2013; Borovsky et al. 2004; Povero et al., 2011). However, a study demonstrated that CHS is up-regulated in fruits of the anthocyanin-pigmented tomato *Aft/Aft* mutant (accession number LA1996) compared with the red-fruited varieties (Sapir et al., 2008). We observed this same expression pattern for the expression of CHS in peel; higher expression in Micro-Tom *Aft/atv/hp2* than in Micro-Tom WT (Supplementary material 3). Furthermore, we observed that the CHS expression was higher in peel compared with flesh in the triple mutant *Aft/atv/hp2* (Figure 6a), the same expression pattern observed by Kang et al. (2018). The CHS is the initial key enzyme of flavonoid biosynthesis, therefore, this different expression between peel and flesh was expected, since this genotype has high anthocyanin accumulation in peel and no accumulation in flesh.

In tomato, the miR9471 was first identified by a degradome sequencing of root and upground tissues of drought-sensitive (*S. lycopersicum*) and drought-tolerant (*S. lycopersicum var. cerasiforme*) tomato genotypes. According to deep sequencing results, the expression level of miR9471a-5p was up-regulated in sensitive above-ground tissues and down-regulated in tolerant above-ground tissues (Candar-Cakir et al., 2016). Our target prediction indicated the CHS gene as a target of miR9471b-3p. The high expression of this miRNA observed by the RT-qPCR analyzes (Figure 5) indicate that this miRNA is present in the tomato

metabolism and possible regulate the anthocyanin biosynthesis pathway by the regulation of the CHS.

The validation of this miRNA target prediction was also performed by CRISPR-Cas9 in Micro-Tom WT. The transformed explants with the vector pDIRECT_22C_miR9471 and pDIRECT_22C_miR9471b designed to knockout the miR9471a / miR9471b precursors and just miR9471b precursor, respectively, were the ones that showed the best results, displaying purple leaf veins and stems (Figure 8 and 9) indicating a high anthocyanin production. The knockout of the miR9471a / miR9471b seems to be more efficient than just miR9471b, since the transformed explants for it showed a stronger purple phenotype. The CHS is not only related with the anthocyanin pathway but also with flavonoid pathway in general, therefore our discovery opens new possibilities of studies with the CHS in the flavonoids pathway, by the knockout of the miR9471.

## CONCLUSION

We performed a set of experiments including, miRNA target prediction, RT-qPCR and CRISPR-Cas9 to better understand the roles of miRNAs on the anthocyanin biosynthesis pathway. The knockout of each miRNA in Micro-Tom WT leaded to a purple phenotype especially in leaf veins and stem at transformed explants. This strongly suggests that the miR5368, miR6024 and miR9471 display negative regulation in the anthocyanin biosynthesis pathway acting in the genes ANS, SlAN1 and CHS, respectively. The manipulation of the miRNAs in plants is a promising approach to improve different desirable characteristic in the plant metabolism. Our findings will open new possibilities for studies with the miRNAs related with the other regulatory and structural genes of the anthocyanin biosynthesis pathway and aiming at an increased anthocyanin accumulation in tomato fruits.

## CONCLUSIONS AND PERSPECTIVES

We observed that miRNAs act on the anthocyanin biosynthesis pathway and influence directly the expression of the genes in this pathway. Here we predicted that some miRNAs act in anthocyanin related genes, among them, *ANTHOCYANIDIN SYNTHASE* (ANS), SlAN1 and *CHALCONE SYNTHASE* (CHS) being potentially regulated by miR5368, miR6024-3p, and miR9471b-3p, respectively. The validation of these three miRNAs was made by RT-qPCR and all of them showed high expression in both Micro-Tom genotypes. Furthermore, we generated the knockout of each miRNAs precursors in Micro-Tom Wild-Type by CRISPR-Cas9. Our results indicate that the miRNAs was knocked-out from the tomato genome, since the transformed explants showed a purple phenotype in leaf veins and stem. This strongly suggests that the miR5368, miR6024 and miR9471 display negative regulation in the anthocyanin biosynthesis pathway acting in the genes ANS, SlAN1 and CHS respectively.

Future analyses are needed to confirm the miRNAs knockout from the tomato genome. Expression analyses in the knockout plants are also needed to verify the increase of the target genes transcripts, as well as the subsequent key biosynthetic genes of the pathway. Anthocyanin quantification is also needed. In addition, the knockout of these miRNAs could lead to some effect in the anthocyanin biosynthesis in tomato fruits.

Our findings contribute to understand the role of some miRNAs on the anthocyanin biosynthesis pathway, thus opening new possibilities for the study of miRNAs related to regulatory and structural genes of the anthocyanin biosynthesis pathway using this approach and aiming at the increase of the anthocyanin accumulation in tomato fruits.

## REFERENCES

ALBERT, Nick W. et al. Light-induced vegetative anthocyanin pigmentation in Petunia. **Journal of experimental botany,** v. 60, n. 7, p. 2191-2202, 2009.

ALBERT, Nick W. et al. A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. **The Plant Cell,** v. 26, n. 3, p. 962-980, 2014.

ANDRÉ, Christelle M. et al. Gene expression changes related to the production of phenolic compounds in potato tubers grown under drought stress. **Phytochemistry,** v. 70, n. 9, p. 1107-1116, 2009.

AUKERMAN, Milo J.; SAKAI, Hajime. Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. **The Plant Cell,** v. 15, n. 11, p. 2730-2741, 2003.

AZA-GONZALEZ, Cesar et al. Anthocyanin accumulation and expression analysis of biosynthesis-related genes during chili pepper fruit development. **Biologia Plantarum,** v. 57, n. 1, p. 49-55, 2013.

BARRETT, Diane M.; BEAULIEU, John C.; SHEWFELT, Rob. Color, flavor, texture, and nutritional quality of fresh-cut fruits and vegetables: desirable levels, instrumental and sensory measurement, and the effects of processing. **Critical reviews in food science and nutrition,** v. 50, n. 5, p. 369-389, 2010.

BAULCOMBE, David. RNA silencing in plants. **Nature,** v. 431, n. 7006, p. 356-363, 2004.

BOROVSKY, Yelena et al. The A locus that controls anthocyanin accumulation in pepper encodes a MYB transcription factor homologous to Anthocyanin2 of Petunia. **Theoretical and Applied Genetics,** v. 109, n. 1, p. 23-29, 2004.

BUER, Charles S.; DJORDJEVIC, Michael A. Architectural phenotypes in the transparent testa mutants of Arabidopsis thaliana. **Journal of experimental botany,** v. 60, n. 3, p. 751-763, 2009.

BULGAKOV, Victor P.; AVRAMENKO, Tatiana V.; TSITSIASHVILI, Gurami Sh. Critical analysis of protein signaling networks involved in the regulation of plant secondary metabolism: focus on anthocyanins. **Critical reviews in biotechnology,** v. 37, n. 6, p. 685-700, 2017.

BUTELLI, Eugenio et al. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. **Nature biotechnology,** v. 26, n. 11, p. 1301, 2008.

ČERMÁK, Tomáš et al. A multipurpose toolkit to enable advanced genome engineering in plants. **The Plant Cell,** v. 29, n. 6, p. 1196-1217, 2017.

CANDAR-CAKIR, Bilgin; ARICAN, Ercan; ZHANG, Baohong. Small RNA and degradome deep sequencing reveals drought and tissue specific microRNAs and their important roles in drought-sensitive and drought-tolerant tomato genotypes. **Plant biotechnology journal,** v. 14, n.8, p. 1727-1746, 2016.

CAO, Xue et al. A putative R3 MYB repressor is the candidate gene underlying atroviolacium, a locus for anthocyanin pigmentation in tomato fruit. **Journal of experimental botany,** v. 68, n. 21-22, p. 5745-5758, 2017.

CARDOSO, T. C. de S. et al. New insights into tomato microRNAs. **Scientific Reports,** v. 8, n. 16069, 2018.

CHEN, Xuemei. A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. **Science,** v. 303, n. 5666, p. 2022-2025, 2004.

CHIUMENTI, Michela et al. A Short Indel-Lacking-Resistance Gene Triggers Silencing of the Photosynthetic Machinery Components Through TYLCSV-Associated Endogenous siRNAs in Tomato. **Frontiers in plant science,** v. 9, p. 1470, 2018.

COLANERO, Sara et al. Alternative splicing in the anthocyanin fruit gene encoding an R2R3 MYB transcription factor affects anthocyanin biosynthesis in tomato fruits. **Plant Communications,** v. 1, n. 1, p. 100006, 2020.

CUI, Long-Gang et al. The miR156-SPL 9-DFR pathway coordinates the relationship between development and abiotic stress tolerance in plants. The Plant Journal, v. 80, n. 6, p. 1108-1117, 2014.

DAI, Xinbin; ZHUANG, Zhaohong; ZHAO, Patrick Xuechun. psRNATarget: a plant small RNA target analysis server (2017 release). **Nucleic acids research,** v. 46, n. W1, p. W49-W54, 2018.

DUBOS, Christian et al. MYB transcription factors in Arabidopsis. **Trends in plant science,** v. 15, n. 10, p. 573-581, 2010.

FAO. (2015). Food and Agriculture Organization of the United Nations. **FAO Statistical Yearbook 2015:** World Food and Agriculture (United Nations, 2015).

FALCONE FERREYRA, Maria Lorena; RIUS, Sebastián; CASATI, Paula. Flavonoids: biosynthesis, biological functions, and biotechnological applications. **Frontiers in plant science**, v. 3, p. 222, 2012.

FILGUEIRA, F. **Solanáceas:** Lavras: Editora UFLA, 2003.

GEORGIEVA, D. et al. Analytical features of an optimized method for HPLC analysis of some polyphenolic acids and flavonoids in tomato fruits. **Agricultural Science and Technology,** v. 6, n. 4, p. 480-485, 2014.

GONZALEZ, Antonio et al. Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. **The Plant Journal,** v. 53, n. 5, p. 814-827, 2008.

GOU, Jin-Ying et al. Negative regulation of anthocyanin biosynthesis in Arabidopsis by a miR156-targeted SPL transcription factor. **The Plant Cell,** v. 23, n. 4, p. 1512-1522, 2011.

GOULD, Kevin S. Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. **BioMed Research International,** v. 2004, n. 5, p. 314-320, 2004.

GUAN, Xueying et al. miR828 and miR858 regulate homoeologous MYB2 gene functions in Arabidopsis trichome and cotton fibre development. **Nature communications,** v. 5, n. 1, p. 1-14, 2014.

HABANOVA, Marta et al. Intake of bilberries (Vaccinium myrtillus L.) reduced risk factors for cardiovascular disease by inducing favorable changes in lipoprotein profiles. **Nutrition research,** v. 36, n. 12, p. 1415-1422, 2016.

HARBORNE, Jeffrey B.; WILLIAMS, Christine A. Advances in flavonoid research since 1992. **Phytochemistry,** v. 55, n. 6, p. 481-504, 2000.

HE, Kai et al. Evaluation of antidiabetic potential of selected traditional Chinese medicines in STZ-induced diabetic mice. **Journal of ethnopharmacology,** v. 137, n. 3, p. 1135-1142, 2011.

HSIEH, Li-Ching et al. Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. **Plant physiology,** v. 151, n. 4, p. 2120-2132, 2009.

JAAKOLA, Laura. New insights into the regulation of anthocyanin biosynthesis in fruits. **Trends in plant science,** v. 18, n. 9, p. 477-483, 2013.

JIA, Xiaoyun et al. Small tandem target mimic-mediated blockage of microRNA858 induces anthocyanin accumulation in tomato. **Planta,** v. 242, n. 1, p. 283-293, 2015.

JONES-RHOADES, Matthew W.; BARTEL, David P.; BARTEL, Bonnie. MicroRNAs and their regulatory roles in plants. **Annu. Rev. Plant Biol.,** v. 57, p. 19-53, 2006.

JUNG, Jae-Hoon et al. The GIGANTEA-regulated microRNA172 mediates photoperiodic flowering independent of CONSTANS in Arabidopsis. **The Plant Cell,** v. 19, n. 9, p. 2736-2748, 2007.

KANG, Song-I. et al. Expression of anthocyanin biosynthesis-related genes reflects the peel color in purple tomato. **Horticulture, Environment, and Biotechnology,** v. 59, n. 3, p. 435-445, 2018.

KETAO, W. A. N. G. et al. Identification and characterization of microRNA during Bemisia tabaci infestations in Solanum lycopersicum and Solanum habrochaites. **Horticultural Plant Journal,** v. 4, n. 2, p. 62-72, 2018.

KHOO, Hock Eng et al. Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. **Food & nutrition research,** v. 61, n. 1, p. 1361779, 2017.

KULCHESKI, Franceli R. et al. Identification of novel soybean microRNAs involved in abiotic and biotic stresses. **BMC genomics,** v. 12, n. 1, p. 307, 2011.

LEI, Kai-Jian et al. Modulation of the phosphate-deficient responses by microRNA156 and its targeted SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 in Arabidopsis. **Plant and Cell Physiology,** v. 57, n. 1, p. 192-203, 2016.

LI, Feng et al. MicroRNA regulation of plant innate immune receptors. **Proceedings of the National Academy of Sciences,** v. 109, n. 5, p. 1790-1795, 2012.

LI, Shaoxuan et al. Identification and characterization of Prunus persica miRNAs in response to UVB radiation in greenhouse through high-throughput sequencing. **BMC genomics,** v. 18, n. 1, p. 938, 2017.

LI, Yue et al. Identification of drought-responsive microRNAs from roots and leaves of alfalfa by high-throughput sequencing. **Genes,** v. 8, n. 4, p. 119, 2017.

LI, Wei et al. Transcriptional regulation of Arabidopsis MIR168a and argonaute1 homeostasis in abscisic acid and abiotic stress responses. **Plant physiology,** v. 158, n. 3, p. 1279-1292, 2012.

LIU, Ying et al. Anthocyanin biosynthesis and degradation mechanisms in Solanaceous vegetables: a review. **Frontiers in chemistry,** v. 6, p. 52, 2018.

LOPEZ-GOMOLLON, Sara et al. Diverse correlation patterns between microRNAs and their targets during tomato fruit development indicates different modes of microRNA actions. **Planta,** v. 236, n. 6, p. 1875-1887, 2012.

LUAN, Yushi; WANG, Weichen; LIU, Ping. Identification and functional analysis of novel and conserved microRNAs in tomato. **Molecular biology reports,** v. 41, n. 8, p. 5385-5394, 2014.

LUO, Qing-Jun et al. An autoregulatory feedback loop involving PAP1 and TAS4 in response to sugars in Arabidopsis. **Plant molecular biology,** v. 80, n. 1, p. 117-129, 2012.

MALIGEPPAGOL, Manamohan et al. Anthocyanin enrichment of tomato (Solanum lycopersicum L.) fruit by metabolic engineering. **Current Science,** p. 72-80, 2013.

MEISSNER, Rafael et al. A new model system for tomato genetics. **The Plant Journal,** v. 12, n. 6, p. 1465-1472, 1997.

MONTEFIORI, Mirco et al. In the Solanaceae, a hierarchy of bHLHs confer distinct target specificity to the anthocyanin regulatory complex. **Journal of experimental botany,** v. 66, n. 5, p. 1427-1436, 2015.

OLSEN, Kristine M. et al. Temperature and nitrogen effects on regulators and products of the flavonoid pathway: experimental and kinetic model studies. **Plant, Cell & Environment,** v. 32, n. 3, p. 286-299, 2009.

ONYILAGHA, Joseoh C.; GROTEWOLD, Erich. The biology and structural distribution of surface flavonoids. **The Ohio State University,** 2004.

ORI, Naomi et al. Regulation of LANCEOLATE by miR319 is required for compound-leaf development in tomato. **Nature genetics,** v. 39, n. 6, p. 787-791, 2007.

PAN, Changtian et al. Identification and expression profiling of microRNAs involved in the stigma exsertion under high-temperature stress in tomato. **BMC genomics,** v. 18, n. 1, p. 843, 2017.

PEER, Wendy Ann; MURPHY, Angus S. Flavonoids and auxin transport: modulators or regulators?. **Trends in plant science,** v. 12, n. 12, p. 556-563, 2007.

PETRONI, Katia; TONELLI, Chiara. Recent advances on the regulation of anthocyanin synthesis in reproductive organs. **Plant science,** v. 181, n. 3, p. 219-229, 2011.

PFAFFL, Michael W. A new mathematical model for relative quantification in real-time RT–PCR. **Nucleic acids research,** v. 29, n. 9, p. e45-e45, 2001.

PINO, Lilian E. et al. The Rg1 allele as a valuable tool for genetic transformation of the tomato 'Micro-Tom' model system. **Plant methods,** v. 6, n. 1, p. 23, 2010.

POJER, Elisa et al. The case for anthocyanin consumption to promote human health: a review. **Comprehensive Reviews in Food Science and Food Safety,** v. 12, n. 5, p. 483-508, 2013.

POVERO, Giovanni et al. Transcriptional analysis in high-anthocyanin tomatoes reveals synergistic effect of Aft and atv genes. **Journal of plant physiology,** v. 168, n. 3, p. 270-279, 2011.

QIU, Zhengkun et al. The tomato Hoffman's anthocyaninless gene encodes a bHLH transcription factor involved in anthocyanin biosynthesis that is developmentally regulated and induced by low temperatures. **PloS one,** v. 11, n. 3, 2016.

RAHIM, Md Abdur; BUSATTO, Nicola; TRAINOTTI, Livio. Regulation of anthocyanin biosynthesis in peach fruits. **Planta,** v. 240, n. 5, p. 913-929, 2014.

RAJAGOPALAN, Ramya et al. A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana. **Genes & development,** v. 20, n. 24, p. 3407-3425, 2006.

RHOADES, Matthew W. et al. Prediction of plant microRNA targets. **Cell,** v. 110, n. 4, p. 513-520, 2002.

SALINAS, María et al. Genomic organization, phylogenetic comparison and differential expression of the SBP-box family of transcription factors in tomato. **Planta,** v. 235, n. 6, p. 1171-1184, 2012.

SAPIR, Maya et al. Molecular aspects of Anthocyanin fruit tomato in relation to high pigment-1. **Journal of Heredity,** v. 99, n. 3, p. 292-303, 2008.

SESTARI, Ivan et al. Near-isogenic lines enhancing ascorbic acid, anthocyanin and carotenoid content in tomato (Solanum lycopersicum L. cv Micro-Tom) as a tool to produce nutrient-rich fruits. **Scientia Horticulturae,** v. 175, p. 111-120, 2014.

SHIM, Seong Hee et al. Ginkgo biloba extract and bilberry anthocyanins improve visual function in patients with normal tension glaucoma. **Journal of medicinal food,** v. 15, n. 9, p. 818-823, 2012.

SPELT, Cornelis et al. anthocyanin1 of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. **The Plant Cell,** v. 12, n. 9, p. 1619-1631, 2000.

STRACKE, Ralf et al. Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. **The Plant Journal,** v. 50, n. 4, p. 660-677, 2007.

VARKONYI-GASIC, Erika et al. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. **Plant methods,** v. 3, n. 1, p. 12, 2007.

KETAO, W. A. N. G. et al. Identification and characterization of microRNA during Bemisia tabaci infestations in Solanum lycopersicum and Solanum habrochaites. **Horticultural Plant Journal,** v. 4, n. 2, p. 62-72, 2018.

WEI, Chunhua et al. The I2 resistance gene homologues in Solanum have complex evolutionary patterns and are targeted by miRNAs. **BMC genomics,** v. 15, n. 1, p. 743, 2014.

WILLIAMS, Christine A.; GRAYER, Renée J. Anthocyanins and other flavonoids. **Natural product reports,** v. 21, n. 4, p. 539-573, 2004.

XIA, Rui et al. Apple miRNAs and tasiRNAs with novel regulatory networks. **Genome biology,** v. 13, n. 6, p. R47, 2012.

XU, Wenjia; DUBOS, Christian; LEPINIEC, Loïc. Transcriptional control of flavonoid biosynthesis by MYB–bHLH–WDR complexes. **Trends in plant science,** v. 20, n. 3, p. 176-185, 2015.

YANG, Fengxi et al. Overexpression of microRNA828 reduces anthocyanin accumulation in Arabidopsis. **Plant Cell, Tissue and Organ Culture (PCTOC),** v. 115, n. 2, p. 159-167, 2013.

YIN, Zujun et al. Identification of conserved microRNAs and their target genes in tomato (Lycopersicon esculentum). **Gene,** v. 414, n. 1-2, p. 60-66, 2008.

ZHANG, Jianguang et al. Identification of conserved microRNAs and their targets from Solanum lycopersicum Mill. **Gene,** v. 423, n. 1, p. 1-7, 2008.

ZHANG, Yang et al. Anthocyanins double the shelf life of tomatoes by delaying overripening and reducing susceptibility to gray mold. **Current Biology,** v. 23, n. 12, p. 1094-1100, 2013.

ZHAO, Daqiu et al. Overexpression of herbaceous peony miR156e-3p improves anthocyanin accumulation in transgenic Arabidopsis thaliana lateral branches. **3 Biotech,** v. 7, n. 6, p. 379, 2017.

**Appendix A**

**Supplementary Table 1.** Sequences of the primers used in the RT-qPCR analysis.

| Primer name | Primer Sequence 5'-3' |
|---|---|
| **FW_ANS** | AAGGAGGATGAGCAGGATG |
| **RV_ANS** | CAGATTCTTCAGCAGGAACAT |
| **Fw_SlAN1** | TGTCCGTACAAAGAAGGGT |
| **Rv_SlAN1** | TCAGCCAATAAGAGTCCAGT |
| **Fw_CHS** | GCAACAAAAATACACCAAGACA |
| **Rv_CHS** | CCTTACGATACTCCTCCACG |
| **Fw_SlJAF13** | GTTTTGTCGCCAATCAAGAG |
| **Rv_SlJAF13** | TCAAGGAATTATTCGCACCA |
| **RT_sly-miR6024-3p** | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGTAAA |
| **Fw_sly-miR6024-3p** | AGGTACATTTTAGCAAGAGTTGT |
| **RT_sly-miR7696c-3p** | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAAGT |
| **Fw_sly-miR7696c-3p** | GGTGTGGGTTTTGAATTATTAGA |
| **RT_sly-miR5368** | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTCTA |
| **Fw_sly-miR5368** | AGAAGTGGACAGTCTCAGG |
| **RT_sly-miR9471b-3p** | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGTGA |
| **Fw_sly-miR9471b-3p** | ATATTTTGGCTGAGTGAGCA |
| **Reverse Universal** | GTGCAGGGTCCGAGGT |
| **Fw_Solanum U6** | GGACATCCGATAAAATTGG |
| **Rv_Solanum U6** | GATTTGTGCGTGTCATCCT |
| **Fw_5.8 S** | GGGCGAGTCCAAAATCCAAT |
| **Rv_5.8 S** | GGTGTTTTCACGTCTTACCGT |

**Appendix B**

**Supplementary Table 2.** Mature and precursor miRNAs sequences and their position in the genome.

| miRNA | miRNA Precursor Sequence | Genome Position | Mature miRNA Sequence |
|---|---|---|---|
| **miR5368** | CUAACCUUGUGUCAGGACCUAUGGGCCAAGGGACA GUCUCAGGUAGACAGUUUCUAUGGGGCGUAGGCC UCCCAAAAGGUAAC | SL2.50ch08 (47023226..47023308) Plus | GGACAGUCUCAGGUAGACA |
| **miR6024** | AACUGGAAAUGGGGAAGUGGAGAAACAACACUUG CUAAAGGAAGUUCACCAGUCAUCUCUUUUCUUUU GGAGUUUUUUUAGCAAGAGUUGUUUUACCUCUUC UCAAU | SL2.50ch01 (83111141..83111026) Minus | UUUUAGCAAGAGUUGUUUUACC |
| **miR9471b** | UUGAGAUUCAGUUGAUUUCUGAGGUGCUCACUCA GCUAAUAGUUAUUGUUUAAGAAACUCAAUAAUAU UGGCAGCAAGGAGAAUGGUGACUUUCAGGAUGAU AACUAUUGGCUGAGUGAGCAUCACUGAAAUCGAC AUGAUUCUGAG | SL2.50ch12 (1979536..1979390) Minus | UUGGCUGAGUGAGCAUCACUG |
| **miR9471a** | CUGAGAUUCAGUUGAUUUCUCAGGUGCUCACUCA GCUAAUAGUUAUUAUUUAAGAAACUCAAUAAUAU UGGCAGCAAGGAGAAUGGUGACUUUCAGGAUGAU AACUAUUGGCUGAGUGAGCAUCACGGAAAUCGAC GUGAUUCUGAG | SL2.50ch12 (1977891..1977745) Minus | UUGGCUGAGUGAGCAUCACGG |

**Appendix C**
**Supplementary Table 3.** gRNAs sequences.

| gRNA | gRNA sequence 5'-3' |
|---|---|
| **gRNA1_miR5368** | AATTCGGTCCATATCCGGCCTGG |
| **gRNA2_miR5368** | ACTGATGGCTCGGGCCCCCCCGG |
| **gRNA1_miR6024** | TAGCTGCAGTTGTCATTCTAGGG |
| **gRNA2_miR6024** | TCCGACCACCGTTCAATCATCGG |
| **gRNA1_miR9471b** | TGAGATTCAGTTGATTTCTGAGG |
| **gRNA2_miR9471b** | GACTCTGTGAAATGATTTGATGG |
| **gRNA1_miR9471** | TGAGATTCAGTTGATTTCTGAGG |
| **gRNA2_miR9471** | TATTGGCTGAGTGAGCATCACGG |

**Appendix D**

**Supplementary Table 4.** Specific primers used to generate the gRNAs fragments by PCR reaction to produce the vectors.

| Identification | Sequence 5'-3' |
|---|---|
| **oCmYLCV_miR5368** | TGCTCTTCGCGCTGGCAGACATACTGTCCCAC |
| **CSY_gRNA1_miR5368** | TCGTCTCCATGGACCGAATTCTGCCTATACGGCAGTGAAC |
| **REP_gRNA1_miR5368** | TCGTCTCACCATATCCGGCCGTTTTAGAGCTAGAAATAGC |
| **CSY_gRNA2_miR5368** | TCGTCTCCCGAGCCATCAGTCTGCCTATACGGCAGTGAAC |
| **REP_gRNA2_miR5368** | TCGTCTCACTCGGGCCCCCCGTTTTAGAGCTAGAAATAGC |
| **CSY_term_miR5368** | TGCTCTTCTGACCTGCCTATACGGCAGTGAAC |
| **oCmYLCV_miR6024-3p** | TGCTCTTCGCGCTGGCAGACATACTGTCCCAC |
| **CSY_gRNA1_miR6024-3p** | TCGTCTCCCAACTGCAGCTACTGCCTATACGGCAGTGAAC |
| **REP_gRNA1_miR6024-3p** | TCGTCTCAGTTGTCATTCTAGTTTTAGAGCTAGAAATAGC |
| **CSY_gRNA2_miR6024-3p** | TCGTCTCCACGGTGGTCGGACTGCCTATACGGCAGTGAAC |
| **REP_gRNA2_miR6024-3p** | TCGTCTCACCGTTCAATCATGTTTTAGAGCTAGAAATAGC |
| **CSY_term_miR6024-3p** | TGCTCTTCTGACCTGCCTATACGGCAGTGAAC |
| **oCmYLCV_miR9471b-3p** | TGCTCTTCGCGCTGGCAGACATACTGTCCCAC |
| **CSY_gRNA1_miR9471b-3p** | TCGTCTCCCATCCTGAAAGTCTGCCTATACGGCAGTGAAC |
| **REP_gRNA1_miR9471b-3p** | TCGTCTCAGATGATAACTATGTTTTAGAGCTAGAAATAGC |
| **CSY_gRNA2_miR9471b-3p** | TCGTCTCCTTTCACAGAGTCCTGCCTATACGGCAGTGAAC |
| **REP_gRNA2_miR9471b-3p** | TCGTCTCAGAAATGATTTGAGTTTTAGAGCTAGAAATAGC |
| **CSY_term_miR9471b-3p** | TGCTCTTCTGACCTGCCTATACGGCAGTGAAC |
| **oCmYLCV_miR9471** | TGCTCTTCGCGCTGGCAGACATACTGTCCCAC |
| **CSY_gRNA1_miR9471** | TCGTCTCCAACTGAATCTCACTGCCTATACGGCAGTGAAC |
| **REP_gRNA1_miR9471** | TCGTCTCAAGTTGATTTCTGGTTTTAGAGCTAGAAATAGC |
| **CSY_gRNA2_miR9471** | TCGTCTCCACTCAGCCAATACTGCCTATACGGCAGTGAAC |
| **REP_gRNA2_miR9471** | TCGTCTCAGAGTGAGCATCAGTTTTAGAGCTAGAAATAGC |
| **CSY_term_miR9471** | TGCTCTTCTGACCTGCCTATACGGCAGTGAAC |

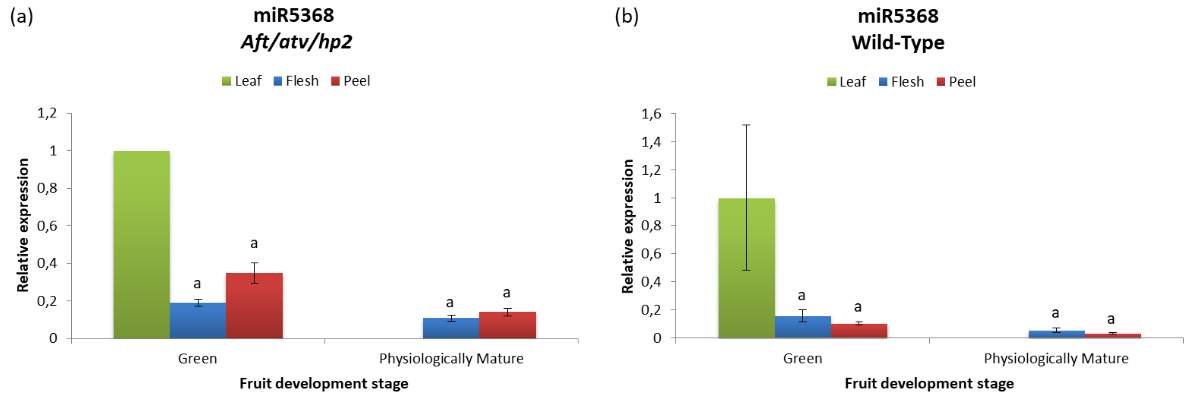
**Appendix E**

**Supplementary Table 5.** miRNA target prediction for the main known anthocyanin biosynthesis related genes in tomato.

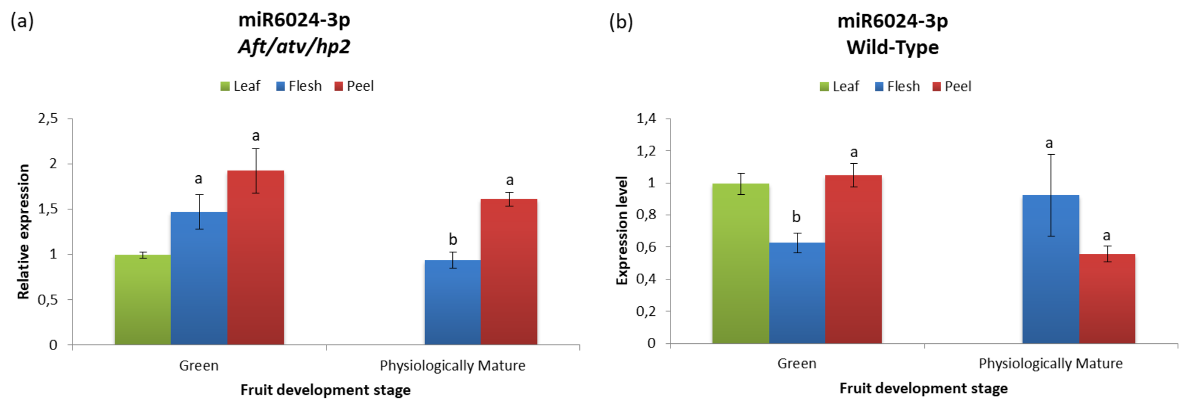
| Gene | Solyc ID | miRNAs | miRNA_aligned_fragment | Inhibition |
|---|---|---|---|---|
| **4CL** | Solyc06g068650 | sly-miR170-1-5p / sly-miR7997a | UAUUGGCCUGGUUC-ACUCAGA / AUGCUGCUCGGACUCUUCAAA | Cleavage / Translation |
| **CHS1** | Solyc09g091510 | sly-miR9471b-3p / sly-miR168a-2-3p | UUGGCUGAGUGAGCAUCACUG / CCUGCCUUGCAUCAACUGAAU | Cleavage |
| **CHS2** | Solyc05g053550 | _ | _ | _ |
| **CHI** | Solyc05g010320 | sly-miR319a-2-3p / sly-miR5282-3p | GACGGAAUUAGAGAGGGAUUUUA / GACGGAAUUAGAGAGGGAUUUUA | Cleavage |
| **CHI-like** | Solyc05g052240 | sly-miR159a-3p | UUUGGAUUGAAGGGAGCUCUA | Cleavage |
| **F3H** | Solyc02g083860 | sly-miR166a-4-5p / sly-miR7981-3p | GGAAUGUUGUCUGGCUCGAGG / AUAGGACUUUAGUUUAGUUAAGGU | Cleavage |
| **F3'5'H** | Solyc11g066580 | sly-miR160g-5p | UGCCUGGCUCCCUGGAUGCCA | Translation |
| **DFR** | Solyc02g085020 | _ | _ | _ |
| **ANS** | Solyc08g080040 | sly-miR5368 | GGACAGUCUCAGGUAGACA | Cleavage |
| **3GT** | Solyc10g083440 | _ | _ | _ |
| **RT** | Solyc09g059170 | sly-miR396b-5p | UUCCACAGCUUUCUUGAACUU | Cleavage |
| **AAC** | Solyc12g088170 | sly-miR394b-3p / sly-miR167a-1-3p | AGGUGGGCAUACUGCCAAUAG / GAUCAUGUGGCAGCCUUACC | Cleavage / Translation |
| **5-GT** | Solyc09g092500 | _ | _ | _ |
| **GST** | Solyc02g081340 | sly-miR172d-2-3p / sly-miR5532-1 | UGAGAAUCUUGAUGAUGCUGCAU / AUGAAAUAUAUGACAAAGGUGG | Cleavage |
| **PAT** | Solyc03g025190 | sly-miR169d-3p / sly-miR408-2-3p | AUGCACUGCCUCUUCCCUGGC / AUGCACUGCCUCUUCCCUGGC | Cleavage |
| **MYB113** | Solyc10g086260 | sly-miR2111a-3p | CCUUGGGAUGCAGAUUAUC | Cleavage |
| **MYB75** | Solyc10g086250 | _ | _ | _ |
| **MYB28** | Solyc10g086270 | _ | _ | _ |
| **MYB114** | Solyc10g086290 | _ | _ | _ |
| **bHLH150** | Solyc09g065100 | sly-miR6024-3p / sly-miR157a-1-3p | UUUUAGCAAGAGUUGUUUUACC / GCUCUUUAUUCUUCUGUCAUCA | Cleavage |
| **bHLH090** | Solyc08g081140 | sly-miR7696c-3p | UUUUGAAUUAUUAGAACUUGA | Translation |
| **AN11** | Solyc03g097340 | _ | _ | _ |

# Appendix F

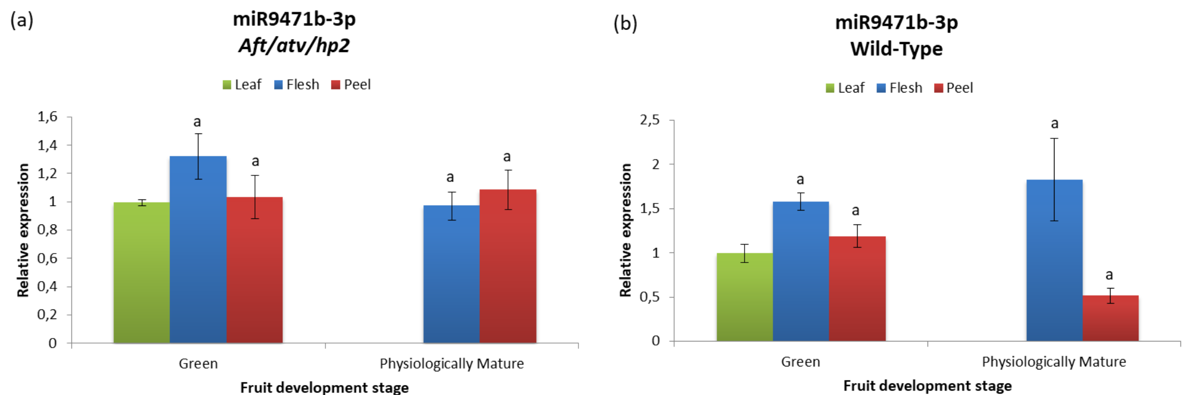
**A**




**B**

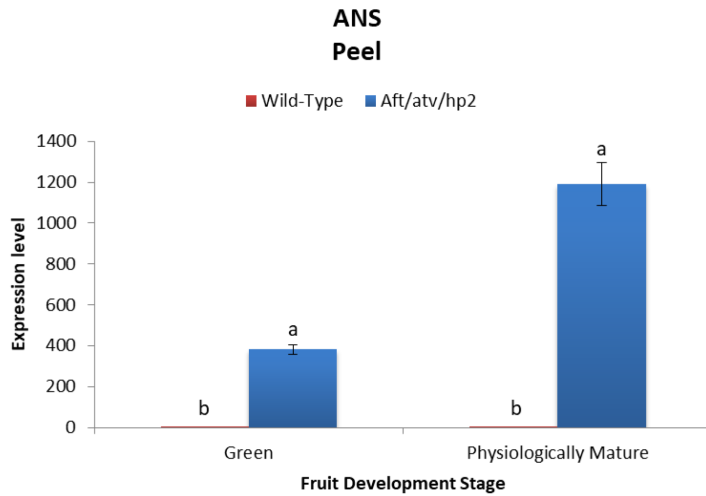



**C**

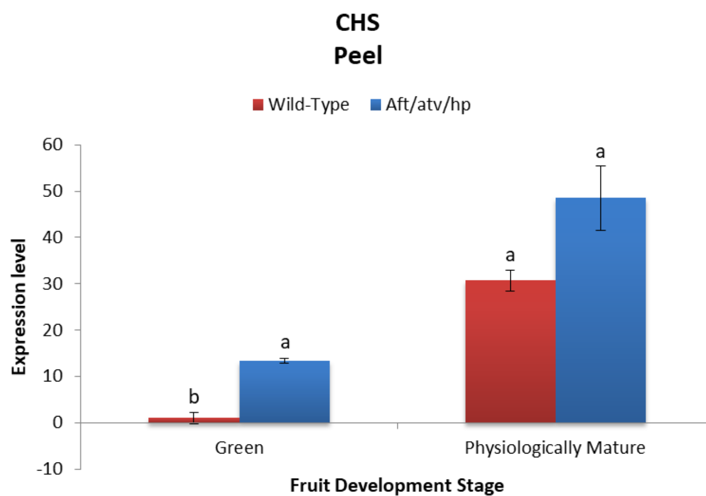



**Supplementary material 1.** Relative expression pattern of miR5368 (A), miR6024-3p (B) and miR9471b-3p (C) in leaf, peel and flesh of two fruits development stage of Micro-Tom *Aft/atv/hp2* (a) and Micro-Tom Wild-Type (b). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.

# Appendix G




**Supplementary material 2.** Relative expression pattern of ANS (Solyc08g080040) in peel of two fruits development stage of Micro-Tom *Aft/atv/hp2* (blue) and Micro-Tom Wild-Type (red). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.




**Supplementary material 3.** Relative expression pattern of CHS (Solyc09g091510) in peel of two fruits development stage of Micro-Tom *Aft/atv/hp2* (blue) and Micro-Tom Wild-Type (red). The transcripts levels are represented as a relative expression between the target gene and reference genes *Solanum* U6 and 5.8S. The data average is of three biological samples.