ADOLFO LUÍS DOS SANTOS

ANÁLISE DA REGULAÇÃO TRANSCRICIONAL DA ROTA METABÓLICA DE ANTOCIANINAS E DE SEUS TRANSPORTADORES EM DIFERENTES TECIDOS DO FRUTO DE TOMATEIRO

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para a obtenção do título de Doutor.

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Santos, Adolfo Luis dos.
Análise da regulação transcricional da rota metabólica de antocianinas e de seus transportadores em diferentes tecidos do fruto de tomateiro / Adolfo Luís dos Santos. - 2017.
221 p. : il.

Orientador(a): Vagner Augusto Benedito.
Coorientador(a): Antônio Chalfun Júnior.
Tese (doutorado) - Universidade Federal de Lavras, 2017.
Bibliografia.

1. Tomate. 2. Antocianina. 3. Antioxidante. I. Benedito, Vagner Augusto. II. Júnior, Antônio Chalfun. III. Título.

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ANALYSIS OF THE TRANSCRIPTIONAL REGULATION OF THE ANTHOCYANIN METABOLIC PATHWAY AND THEIR TRANSPORTERS IN DIFFERENT TOMATO FRUIT TISSUES

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para a obtenção do título de Doutor.

Aprovada em 31 de março de 2017
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LAVRAS – MG
2017

A Deus**,**

DEDICO.

AGRADECIMENTOS

À Universidade Federal de Lavras (UFLA) e ao Programa de Fisiologia Vegetal, por toda minha formação.

Ao meu orientador, professor PhD. Vagner Augusto Benedito, por toda paciência, pelo enorme aprendizado, treinamento e confiança depositada. Obrigado por toda essa oportunidade, com certeza ela será o diferencial na minha carreira profissional.

Ao meu co-orienador PhD, Antônio Chalfun Júnior, por me acolher junto aos seus orientados durante minha estadia no programa.

Aos membros do Laboratório de Fisiologia Molecular de Plantas pelo acolhimento e companheirismo.

A todos os amigos da UFLA que de alguma forma me ajudaram nesta fase. Em especial ao Cardon, Wesley, André, Renan pelas discussões durante os lanches.

Ao casal de amigos Lucas Maia e Dayane pelo acolhimento, amizade e companheirismo durante minha estadia nos EUA e depois.

A minha família, que mesmo não entendendo ao certo o que eu fazia, sempre apoiou. Em especial a minha namorada Pamina que suportou toda minha ausência e meu estresse durante essa etapa.

A *West Virginia University* pela hospitalidade.

A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo financiamento tanto durante minha estadia no Brasil quanto durante meu doutorado sanduíche em EUA.

A todos meu Muito Obrigado!

"A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original"

Albert Einstein

RESUMO

As antocianinas são compostos do metabolismo secundário das plantas e estão inseridos dentro do grupo dos flavonóides. As antocianinas são responsáveis pela pigmentação roxa de frutos e apresentam importantes funções não só para os vegetais, mas também para a saúde humana. Sendo assim, o enriquecimento de antocianinas em frutos torna-se importante, sendo o tomate um candidato ideal para este enriquecimento por estar entre as hortaliças mais consumidas no mundo. Além da engenharia genética, o melhoramento clássico de plantas por meio de cruzamentos de genótipos é uma técnica viável para se conseguir tal enriquecimento. No entanto, é necessário ter conhecimento não só sobre a rota de biossíntese de antocianina em tomates, mas também sobre os genes que regulam esta rota, tal como transportadores desse pigmento na célula. Atualmente já se sabe que em dicotiledôneas a fase inicial da rota de antocianina é regulada por fatores de transcrição R2R3-MYB, enquanto a fase tardia é regulada pelo complexo MYB-bHlH-WD40 (MBW), além disso, transportadores da família MATE (*Multidrug and Toxic Compound Extrusion*) ou ABC (ATP-Binding Cassette), são responsáveis pelo transporte de antocianina no vacúolo. Recentemente, um mutante triplo de tomateiro envolvendo os loci *Aft, atv e hp2* foi gerado. O genótipo apresenta acúmulo de antocianina, o que confere coloração roxa ao epicarpo dos frutos. Trabalhos recentes identificaram por meio de ensaios de RT q-PCR dois fatores de transcrição (*Sl*MYB114 e *Sl*TT8), que provavelmente são responsáveis por regular a biossíntese de antocianina no genótipo *Aft/atv/hp2*. Neste trabalho nós estudamos o acúmulo de antocianina em alimentos carnosos comestíveis, relatamos características do desenvolvimento de plantas do genótipo *Aft/atv/hp2*, caracterizamos a família de proteínas MATE em *Solanum lycopersicum* e apontamos uma provável proteína MATE responsável por transportar antocianina em tomates, além disso, construímos vetores binários para futuras transformações de plantas de tomate afim de comprovar as funções dos genes identificados anteriormente por meio da superexpressão concomitante dos fatores *Sl*TT8 e S*l*MYB114 em tomateiros selvagens de fruto vermelho, bem como a supressão da expressão gênica específica do SlMYB114 em frutos roxos via CRISPR/Cas9. Este trabalho traz dados antes desconhecidos sobre o recente genótipo *Aft/atv/hp2* e sobre proteínas MATE em *Solanum lycopersicum* que servirá de base para novas pesquisas sobre o transporte não só de antocianinas, mas de vários outros compostos importantes para o metabolismo e desenvolvimento tanto do tomateiro, como de outras Solanáceas.

Palavras-chave: Antioxidante. Fitonutriente. Filogenia. Heatmap

ABSTRACT

Anthocyanins are composed of the secondary metabolism of plants and are inserted into the flavonoid group. Anthocyanins are responsible for the purple pigmentation of fruits and present important functions not only for plants but also for human health. Thus, the enrichment of anthocyanins in fruits becomes important, being the tomato an ideal candidate for this enrichment as it is among the most consumed vegetables in the world. In addition to genetic engineering, classical breeding of plants through genotype crosses is a viable technique for achieving such enrichment. However, it is necessary to be aware not only of the route of anthocyanin biosynthesis in tomatoes, but also of the genes that regulate this route, such as transporters of that pigment in the cell. Currently known that in dicotyledons the initial phase of the anthocyanin route is regulated by R2R3-MYB transcription factors, whereas the late phase is regulated by the MYB-bHlH-WD40 (MBW) complex, in addition, MATE (Multidrug and Toxic Compound Extrusion) or ABC (ATP-Binding Cassette), are responsible for the transport of anthocyanin in the vacuole. Recently, a triple tomato mutant involving the *Aft*, *atv* and *hp2* loci was generated. The genotype shows accumulation of anthocyanin, which gives purple coloration to the epicarp of the fruits. Recent works have identified two transcription factors (*Sl*MYB114 and *Sl*TT8), which are probably responsible for regulating anthocyanin biosynthesis in the *Aft / atv / hp2* genotype. In this work we studied the accumulation of anthocyanin in edible fleshy foods, we report characteristics of the development of *Aft / atv / hp2* genotype plants, characterize the MATE family of proteins in *Solanum lycopersicum* and point out a probable MATE protein responsible for transporting anthocyanin in tomatoes, In addition, we constructed binary vectors for future tomato plant transformations in order to prove the functions of genes previously identified by concomitant superexpression of the *Sl*TT8 and *Sl*MYB114 factors in wild red fruit tomatoes, as well as the suppression of the specific gene expression of *Sl*MYB114 in fruits Purples via CRISPR / Cas9. This work brings previously unknown data on the recent *Aft/atv/hp2* genotype and on MATE proteins in *Solanum lycopersicum* that will serve as the basis for new research on the transport not only of anthocyanins but of several other important compounds for the metabolism and development of both the Tomato, as of other Solanaceae.

Keywords: Antioxidant. Phytonutrient. Phylogeny. Heatmap

SUMÁRIO

PARTE 1

1. INTRODUÇÃO

Flavonóides são compostos fenólicos, oriundos do metabolismo secundário das plantas e responsáveis pela maior parte da coloração amarela, alaranjada, vermelha, violeta e azul de flores e frutos. Os flavonóides são comumente encontrados em alimentos como frutas, legumes, flores, vinho, chá, própolis e mel, representando o grupo mais comum de compostos fenólicos na alimentação humana (HICHRI et al., 2010). Atualmente mais de 8000 flavonóides estão descritos, e dentre eles as antocianinas formam um dos grupos químicos mais estudados (GU et al., 2003).

As antocianinas estão presentes em diversas espécies e podem ser encontradas em diferentes partes dos vegetais. A maior concentração do pigmento está geralmente em flores e frutos, onde atuam atraindo organismos polinizadores e dispersores de sementes (SPENCER et al., 2009). Além disso, as antocianinas podem modificar significativamente a quantidade e a qualidade da luz incidente sobre os cloroplastos e assim proteger tecidos fotossintéticos contra fotoinibição e a formação de espécies reativas de oxigênio (ROS) (STEYN et al., 2002).

Atualmente têm-se observado um crescente interesse no uso de pigmentos antociânicos em diversos segmentos, dentre os quais se destacam as indústrias cosméticas, alimentícia e farmacêutica, onde podem ser aplicadas, por exemplo, como corantes naturais, ou utilizadas para o desenvolvimento de novos fármacos e suplementos alimentares, uma vez que as antocianinas apresentam benefícios à saúde devido suas atividades biológicas, que incluem propriedades antioxidantes e inflamatórias (DIACONEASA et al., 2015), inibição da oxidação do LDL (CHANG et al., 2006), diminuição dos riscos de doenças cardiovasculares (TOUFEKTSIAN et al., 2008), de câncer (BUTELLI et al., 2008; CHAREPALLI et al., 2015), promoção da acuidade visual, além de ajudar na prevenção da obesidade e diabetes (GUO & LING, 2015).

Alguns alimentos têm sido relatados como importantes fontes de antocianina na dieta, dentre eles podem ser citados o açaí (DA SILVA SANTOS et al., 2014), amora (GUEDES et al., 2014), cereja (WEI et al., 2015), bem como uvas escuras (HICHRI et al., 2010) e feijão preto (AZEVEDO et al., 2003). Infelizmente as antocianinas não estão comumente presentes em alimentos importantes como o tomate por exemplo, um dos vegetais mais consumidos no mundo (CARVALHO & PAGLIUCA, 2007). O tomate é um candidato ideal para o enriquecimento de antocianina, além de ser uma das hortaliças mais bem aceitas no mundo, sendo consumido *in natura*, ou de formas processadas, apresenta grande produtividade agronômica e disponibilidade durante todo o ano.

Além da engenharia genética, cruzamentos entre genótipos também têm sido utilizados para a produção de plantas com maiores concentrações de antocianinas. Em ambas as abordagens é importante que se conheçam os genes biossintéticos e os genes reguladores da rota de produção deste pigmento. A rota de biossíntese dos flavonóides acontece no citoplasma e é dividida em três fases, a primeira fase é conhecida como metabolismo geral dos fenilpropanóides, a segunda é conhecida como fase inicial dedicada aos flavonóides, e a terceira é a fase tardia da rota (LI, 2014). Em seguida, proteínas transportadoras da família MATE (*Multidrug and Toxic Compound Extrusion*) ou ABC (ATP-Binding Cassette)(REA, 2007), são responsáveis pelo transporte e acúmulo da antocianinas no vacúolo (ZHAO & DIXON, 2009; PÉREZ-DÍAZ et al., 2014; DARBANI et al., 2016), entretanto, pouco ainda se sabe sobre as proteínas do tipo MATE em *Solanum lycopersicum*. Em dicotiledôneas, os primeiros genes da fase inicial da biossíntese *(Early Biosynthesis Genes – EBGs)* são regulados por fatores de transcrição do tipo R2R3-MYB , enquanto os genes tardios *(Late Biosynthesis Genes – LBGs)* necessitam do complexo ternário MYB-bHLH-WD40 para sua ativação (LI, 2014). Vários estudos já apontaram o complexo MYB-bHLH-WD40 (MBW) como sendo um dos responsáveis pela regulação da síntese de

antocianinas e, consequentemente, a pigmentação roxa em diferentes genótipos e em diferentes órgãos das plantas (LI, 2014; MONTEFIORI et al., 2015; XIE et al., 2016).

Pesquisadores produziram por meio de transgenia um tomate roxo rico em antocianinas, tanto na casca quanto na polpa do fruto, por meio da introdução de dois genes de *Antirrhinum majus*, *DELILA* e *ROSEA1* (das famílias bHLH e R2R3-MYB, respectivamente) (BUTELLI et al., 2008). Ademais, recentemente foi produzido um mutante de tomateiro triplo (*Aft/atv/hp2*) cujos frutos roxos apresentam aumento dos níveis de carotenóides (licopeno), vitamina C (ascorbato) e antocianinas sem observar qualquer perda de produtividade (SESTARI et al., 2014). Apesar do alto nível de pigmentação no epicarpo desse fruto, o mesocarpo não apresenta acúmulo significativo de antocianina. Afim de identificar os genes responsáveis pela regulação da biossíntese de antocianina em tomates mutantes *Aft/atv/hp2,* recentemente por meio de ensaios de qRT-PCR foram identificados genes que transcrevem para os fatores de transcrição bHLH e R3R3-MYB (*Sl*TT8 e *Sl*MYB114, respectivamente), que provavelmente são responsáveis pela pigmentação roxa no epicarpo de tomates roxo *Aft/atv/hp2* (CHAVES, 2015). Porém, é necessário mais pesquisas para comprovar as funções exatas desses genes na regulação da biossíntese de antocianina em genótipos *Aft/atv/hp2,* bem como estudar as proteínas MATE em *Solanum Lycopersicum* e apontar uma provável transportadora de antocianina em frutos desse genótipo.

OBJETIVOS

Os objetivos deste trabalho foram:

1) Estudar o acúmulo de antocianina em alimentos carnosos comestíveis (especificidade tecidual);

2) Relatar características do desenvolvimento de plantas do genótipo *Aft/atv/hp2;*

3) Construir vetores binários para futuras transformações de plantas de tomate afim de comprovar as funções dos genes identificados anteriormente;

4) Caracterizar a família de proteínas MATE em *Solanum lycopersicum*;

5) Apontar uma provável proteína MATE responsável por transportar antocianina em tomates;

O presente trabalho é composto por cinco artigos, cada um com o intuito de cumprir os objetivos descritos acima.

2. CONCLUSÕES E PERSPECTIVAS FUTURAS

De forma geral, podemos concluir que o acúmulo de antocianinas em órgãos vegetais é regulado pelos fatores de transcrição pertencentes ao complexo ternário MBW. Entretanto, ainda são necessários mais estudos com o intuito de descobrir os detalhes genético-moleculares da regulação da expressão desses genes em tecidos específicos em órgãos comestíveis de espécies horticulturais. Já se sabe que em laranjas sanguíneas e em maçãs que acumulam antocianina na polpa do fruto, este acumulo ocorre devido a rearranjos na região promotora do fator de transcrição MYB (RUBY e MdMYB10, respectivamente). Esse conhecimento serve como evidência para pesquisas futuras afim de entender essa complexa regulação da biossíntese de antocianina em diferentes tecidos das plantas.

O recente desenvolvimento do genótipo *Aft/atv/hp2* traz entusiasmo aos pesquisadores que trabalham com biossíntese de antocianinas, pois possui características peculiares. Sabe-se que o acúmulo de antocianinas em frutos acontece no decorrer de sua maturação, entretanto, no genótipo *Aft/atv/hp2* o acúmulo de antocianina acontece desde o início do desenvolvimento dos frutos, sendo este fato devido ao estímulo luminoso, o que condiz com a literatura e com os experimentos realizados neste trabalho.

As clonagens genéticas e construções de vetores descritas nesse trabalho abrem o caminho para transformações de tomateiros para o estudo das funções dos genes *Sl*TT8 e *Sl*MYB114 na regulação da biossíntese de antocianina em tomates.

Anteriormente a este trabalho, pouco se conhecia sobre as proteínas transportadoras da família MATE em *S. lycopersicum*. Este trabalho trouxe a

identificação de 67 membros da família MATE no genoma do tomateiro. Esse estudo servirá de base para novas pesquisas sobre o transporte não só de antocianinas, mas de vários outros compostos importantes para o metabolismo e desenvolvimento tanto do tomateiro, como de outras Solanáceas. Ademais, o trabalho trouxe uma análise compreensiva *in silico* da expressão de todos membros MATE em *S. lycopersicum*.

Na continuação deste estudo, a confirmação dos papéis de genes-chaves na regulação da síntese de antocianinas especificamente em tecidos do fruto do tomateiro se faz necessária por meio da superexpressão concomitante dos fatores SlTT8 e SlMYB114 em tomateiros selvagens de fruto vermelho, bem como a supressão da expressão gênica específica do SlMYB114 em frutos roxos via CRISPR/Cas9. Ademais, a identificação de possíveis reguladores mestres dos reguladores imediatos da rota, bem como inibidores da expressão dos genes estruturais e reguladores, poderá ajudar a elucidar as redes genética de regulação dessa rota metabólica, bem como contribuir com ferramentas para o melhoramento nutritivo de frutos carnosos.

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PARTE 2: Artigos

ARTIGO 1: Understanding the genetic regulation of anthocyanin metabolism for boosting antioxidant contents in fruits and vegetables

Submitted to: Jornal of the Science of Food and Agriculture

Understanding the genetic regulation of anthocyanin metabolism for boosting antioxidant contents in fruits and vegetables

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Running title: Understanding anthocyanin metabolism regulation to enhance antioxidant content in produce

Understanding the genetic regulation of anthocyanin metabolism for boosting antioxidant contents in fruits and vegetables

ABSTRACT

Anthocyanins are naturally occurring flavonoid molecules derived from the phenylpropanoid pathway. In addition to their physiological activities in plants, scientific evidence is mounting that the dietary consumption of these plant metabolites offers health benefits. There is increasing evidence of the preventative and protective roles of anthocyanins against a broad range of pathologies, including different cancer types and metabolic diseases. However, most of the fresh produce available to consumers typically contains limited amounts of anthocyanins. Cyanic varieties have often the accumulation of this pigment restricted only to epidermal layers (skin) of plant structures. Therefore, transgenic and non-transgenic approaches have been proposed to enhance the levels of this phytonutrient in vegetables, fruits, and cereals. Here, we reviewed the available evidence of health-promoting activities of dietary anthocyanin consumption in model systems, and cover the current literature on the anthocyanin biosynthesis pathway in model and crop species, including structural and regulatory genes involved in the differential pigmentation patterns of plant organs. Furthermore, we explored the genetic regulation of anthocyanin biosynthesis and the reasons

why it is strongly repressed in specific cell types, in order to create more efficient strategies to boost the biosynthesis and accumulation of anthocyanins in fresh fruits and vegetables.

Keywords: antioxidants; functional food; health; nutraceutical; nutrient-dense food; plant breeding.

INTRODUCTION

Anthocyanins are secondary metabolites of the phenylpropanoid pathway that are widely distributed in the plant kingdom. These glycosylated polyphenols are one of the most important water-soluble pigments in plants[1] and provide the shades of blue, purple, red and pink to plant organs, including leaves, petals, fruits, and seeds[2, 3]. Notwithstanding, it important that a noticeable exception is the order Caryophyllales, which species synthesize indole alkaloid pigments called betalains instead. Common examples of red and purple plants are beet, chard and amaranth (Amaranthaceae), the false Christmas cactus (*Schlumbergera truncata*, Cactaceae), and purslane (Portulacaceae).

Anthocyanins produce visual cues for pollination, and many defense systems against biotic (insect attacks and diseases) and abiotic stresses (e.g., drought, salt, cold, high light intensities, and UV radiation)[4]. Additionally, as non-energetic components of the human nutrition, many studies have highlighted the health benefits, thus helping in the prevention and control of different pathologies. Indeed, there is compelling evidence in the medical literature that these pigments have anti-inflammatory [5, 6], anticancer[7-9], and cardioprotective properties[10], in addition to acting as inhibitors of neurodegeneration[11, 12].

Most importantly, these health benefits are achieved only when a considerable amount of anthocyanins is regularly consumed in the diet[7]. However, most of the vegetables available in the market contain only small quantities of anthocyanins in their edible organs, with the pigment often restricted to epidermal layers (skin), such as leaf and petal epidermis, the coat of seeds (testa), and the epidermal cells of the fruit (exocarp, peel)[7]. Since the peel usually accounts for less than 5% of the total mass of edible parts of the plant, such as the fruit[13], the recalcitrance of parenchymal cells to accumulate anthocyanins in cortical tissues limits considerably the total amount of anthocyanins in most fresh foods available today. Even blueberry, which is popularly regarded as a "super food" due to the antioxidant power derived from anthocyanins[14], the biosynthesis and accumulation of these pigments are exclusively restricted to the exocarp, as we can notice by its white flesh. This differential pigmentation pattern of plant organs showing cyanic epidermis (colored skin/exocarp) and acyanic cortex (e.g., white flesh with non-pigmented parenchymal cells) is commonly found in eggplant, grape, plum, apple, radish, as well as in purple varieties of tomato, onion, and cabbage, to name a few (**Figure 1**). Nonetheless, this is not a universal fact: there are remarkable examples of variant plant genotypes bearing edible organs with cyanic parenchymata, such as purple potatoes, red-flesh apples, *teinturier* grapes, purple carrots, as well as dark-flesh stone fruits (e.g., cherries, peaches, and plums). Therefore, a better understanding of the genetic regulation of anthocyanin

biosynthesis and the reasons why the pathway is repressed in parenchymal cells will lead to efficient ways to breed food for high-density of this phytonutrient in vegetables[15], fruits[13, 16] and cereals[17], potentially with a transformative impact on the health of the consumer population.

Here, despite being considered controversial by some, we start by reviewing the scientific evidence of the effects of anthocyanin-rich diets on human health as the rationale to breeding plants for high anthocyanin content. For that, we further present basic information on the structural and regulatory genes involved in the anthocyanin biosynthesis pathway in plant models as well as crop species. Finally, we focus on the differential patterns of anthocyanin accumulation in tissues and the cell types that comprise the fleshy fruit.

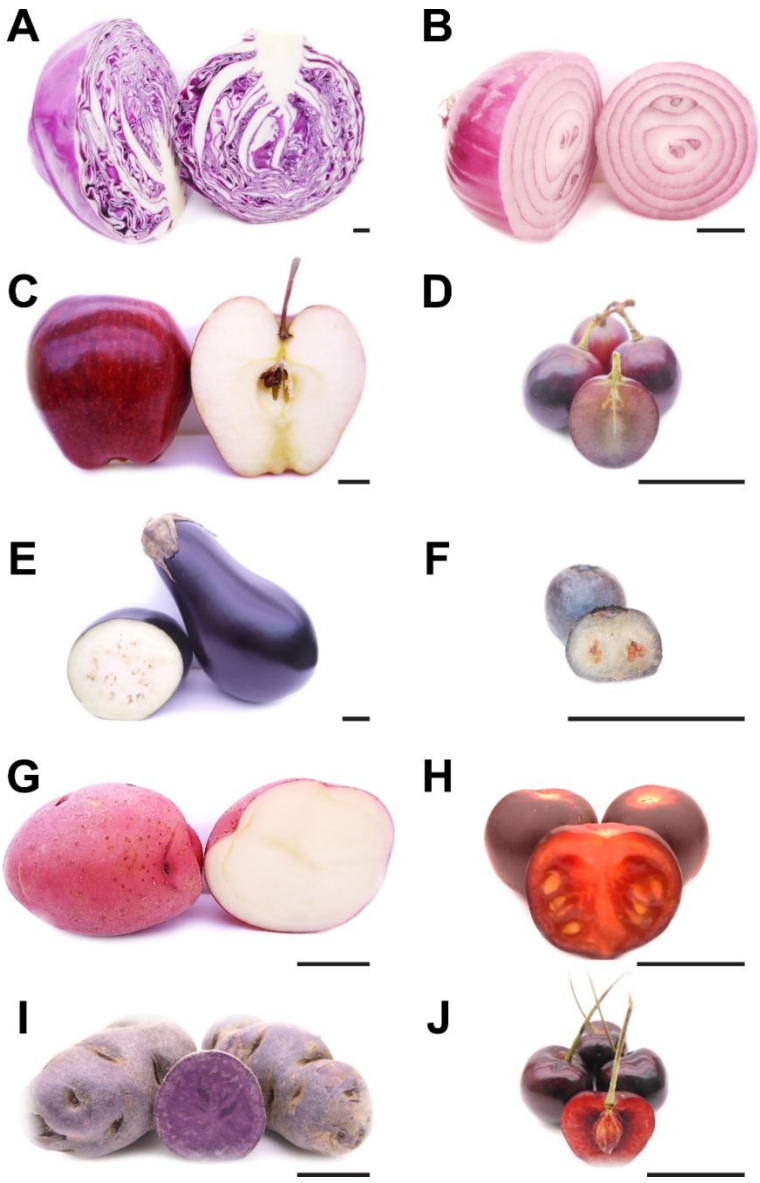

Figure 1. Dietary sources of anthocyanins. (A) Purple cabbage. (B) Onion bulb. (C) Apple. (D) Grape. (E) Eggplant. (F) Blueberry. (G) Potato. (H) Purple tomato (*Aft*/*atv*/*hp2* triple mutant, cv. Micro-Tom[12]). (I) Purple potato. (J) Red cherry.

Except for H-J, all other examples accumulate anthocyanin in the epidermal tissue, while parenchymal cells remain acyanic. Scale: 20 mm

THE RATIONALE FOR BREEDING FOOD WITH HIGH ANTHOCYANIN CONTENT

When consumed in the diet, anthocyanins become highly available in the human organism[18, 19] and activate endogenous antioxidant systems and signaling pathways that delay oxidative damage and progression of malignant tumors[7, 20, 21]. Many studies show a negative correlation between high polyphenol consumption and the incidence of chronic diseases related to the heart[10, 22], obesity[23], blood sugar levels[12] and neuron functions[24]. Since anthocyanins are the most abundant polyphenols in fruits and vegetables[25, 26], 59 completed and 16 ongoing clinical trials studying their bioavailability, pharmacokinetics and health effects in human systems are currently available (www.ClinicalTrials.gov).

Most of the research on the anticancer activity of anthocyanins has used *in vitro* cell assays[25, 27]. The current understanding is that anthocyanins act by multiple (unknown) mechanisms, such as cell cycle arrest (G1/G0 and G2/M)[28], apoptosis induction[29], inhibition of DNA oxidative damage[30], general anti-mutagenesis effects[31], induction of phase II detoxification enzymes[32], and activation of endogenous antioxidant defense systems and signaling pathways[20, 33]. More recently, cyanidin (A18) was shown to repress the signal cascade of the

pro-inflammatory cytokine interleukin-17A (IL-17A), thus preventing the inflammatory responses that lead to common autoimmune inflammatory diseases, which include asthma, rheumatoid arthritis, psoriasis, and cancer[5]. Understandably, the leap from *in vitro* results to clinical effects is often unconvincing. Therefore, *in vitro* studies should not be seen as more than preliminary evidence of potential effects to encourage further research. On the other hand, additional studies have recently used mammal models and human cell cultures to test anti-cancer properties as well as to characterize the molecular mode of action related to *in vivo* properties and activity of anthocyanidins and anthocyanins. *In vitro* and *in vivo* methods were applied to study the effects of the purple-flesh potato (variety Purple Majesty) on cervical cancer. Meristematic cells isolated from primary human colon tumors were exposed to medium supplemented with purple potato extract (5 μg/mL) or sulindac (12.5 μg/mL) as the positive control[8]. Indeed, this non-steroidal drug is effective in eliminating meristem cells with nuclear β-catenin (an indicator of cervical cancer) and also to reduce the number of polyps in *APC*$^{Min/+}$ mice, a well-established model for studies of this type of cancer[34]. Charepalli *et al.*[8] demonstrated that purple potato extract was significantly more effective than sulindac to suppress the proliferation and induce apoptosis of colon cancer cells in *in vitro* cultures. The authors also showed that *ad libitum* AIN-93G diet with 20% (w/w) baked purple potato powder fed to A/J male mice injected with azoxymethane (AOM, a DNA alkylating agent)

to induce colon cancer. The purple potato extract was as effective as the positive control (AIN-93G supplemented with 0.06% sulindac) to suppress tumor incidence after four weeks of treatment.

p53, a tumor suppressor protein in animals, is often called the guardian of the genome due to its normal function as a gatekeeper of the cell cycle progression. This transcription factor is the gene most commonly mutated during tumorigenesis, with a mutation frequency of over 50% in human cancers, including colon cancer[35]. The lack of p53 function leads to uncontrolled cell proliferation, which then progresses to adenomas to carcinomas. Therefore, it is important to test whether strategies developed against the spreading of cancer cells work even in the absence of p53 function.

Accordingly, the anticancer activity of purple potatoes was evaluated in both, cells with normal p53 activity as well as those which the p53 gene expression was attenuated by shRNA lentiviral infection[8]. Interestingly, the anthocyanin-rich purple potato extract significantly suppressed cancer cell proliferation in both, cells with normal p53 function (63% compared to the negative control diet with no supplementation) as well as those with attenuated p53 (32% compared to the negative control diet). In comparison, the positive control treatment (sulindac) suppressed 55% of cells with normal p53 function, while only 16% of meristematic colon cells with attenuated p53 were inhibited by the drug. In

addition to this anti-proliferative activity, the extract also showed pro-apoptotic activity by inducing 28% and 44% more apoptosis in tumor cells with active and attenuated p53, respectively. These results suggest that in colon cancer, the purple potato extract is capable of inhibiting the p53-dependent proliferation of cancer meristematic cells, although this extract may be even more efficient in suppressing tumors with p53-independent progression than the pharmacological control used[8]. The authors found that mice fed diets supplemented with purple potatoes had the Wnt/β-catenin pathway repressed. This signaling pathway directs gene expression programs of embryonic development by directing cell polarity, proliferation, and death[36]. In humans, perturbation of this pathway is invariably linked to congenital disabilities and diseases, including cancer[37, 38]. In colon cancer, the Wnt/β-catenin pathway controls cancer cell proliferation, mutations in the tumor suppressor gene *APC* that leads to higher translocation rates of nuclear β-catenin, which subsequently activates *Wnt* genes, and finally the formation of adenomas. In addition to suppressing this pathway and eliminating cells with β-catenin nuclear, the purple potato supplementation reduced the number of cancer cells by inducing proteins which activities are related to mitochondria-mediated apoptosis (e.g., Bax and Bcl-2)[8]. It is feasible that the anti-inflammatory and anti-cancer activities of anthocyanins start with the repression of IL-17 cytokine signaling cascade[5], thus helping with normal expression of p53[39] and suppression of the Wnt/β-catenin pathway.

Therefore, dietary supplementation with cyanic food proved effective in controlling the proliferation of colon cancer. In four weeks of the experiment, mice that consumed purple potatoes showed a 50% reduction in the incidence of tumors larger than 2mm. Although comparable levels of inhibition were obtained with sulindac, the mice that received this anti-inflammatory drug experienced significant gastrointestinal toxicity (stomach and intestine ulcers) and loss of fat deposits, while this side effect was not observed in any of the subjects that consumed the anthocyanin-rich diet[8].

Another important experiment relating diet supplementation with anthocyanins and cancer suppression was conducted by Butelli and collaborators[7]. Transgenic purple tomatoes were obtained by expressing in the fruit two heterologous transcription factors from *Antirrhinum majus* (*Delila* and *Rosea1*) to boost anthocyanin accumulation[40]. To verify whether anthocyanin-rich tomatoes showed health-promoting properties in a dietary context, the authors tested diets supplemented with purple and red tomatoes in cancer-prone *Trp53*$^{-/-}$ knockout mice. These animals are widely used in bioassays of compounds with anticancer activity because the p53 loss-of-function causes various cancer types to develop spontaneously. Comparing *Trp53*$^{-/-}$ mice fed the standard diet with those fed diets supplemented with powder of the common (red) or transgenic (purple) tomatoes (10% w/w), the authors detected a significant increase in life expectancy of the mice fed the anthocyanin-rich diet. The average lifespan of those fed the standard

diet was 142 days, and the maximum lifespan of 211 days, which is consistent with other reports[41, 42]. For animals fed a diet supplemented with red tomatoes, no significant improvement in longevity was observed, and the mean lifespan of the group was 146 days. However, *Trp53*$^{-/-}$ mice fed the diet supplemented with purple tomato powder had a significantly higher average life expectancy, 182 days, with the maximum lifespan reaching 260 days[7]. Therefore, this experiment strongly suggests that the level of anthocyanins accumulated in the purple tomatoes is sufficient to confer significant protective effect against cancer progression in *Trp53*$^{-/-}$ mice. An important point to note is that in the two studies discussed above[6, 7], rats received the same diet in control and treatment groups, except for anthocyanin-rich food supplementation. This experimental design excludes the possibility that the decrease in tumorigenic frequency, increased lifespan and increased risk of hepatology could be induced or related to other components of the diets.

Furthermore, a study demonstrated that the juice of blood orange (cv Moro), which contains high anthocyanin levels[43], has hepatoprotective properties by combating non-alcoholic fatty liver disease (NAFLD)[44]. NAFLD is a chronic metabolic disease that leads to cardiovascular problems and even death due to liver damage[43, 44]. It is closely associated with obesity, dyslipidemia, diabetes, as well as the spectrum of metabolic syndrome, in which insulin resistance is a common pathophysiological determinant. Recently, the consumption of fructose-

rich foods and beverages has been identified as a major risk factor for NAFLD[45, 46]. However, despite its fructose content, Moro oranges are also rich in anthocyanins, which have been demonstrated to benefit health by modulating the expression of major enzymes involved in glucose sensitivity and lipid homeostasis[47]. In order to analyze the hepatoprotective effect of orange juice intake, three rat groups were provided differentiated diets for 12 weeks. The standard diet (SD) provided 3.3 Kcal/g with 60% carbohydrates, 23% protein and 17% fat. The high-fat diet (HFD) provided 5.2 kcal/g with 20% carbohydrates, 20% protein and 60% fat. The three groups of rats were fed as following: group I (SD + water), group II (HFD + water), and group III (HFD + Moro juice), with *ad libitum* access to the liquid. After 12 weeks, group I and III rats presented the same body weight, while group II rats had significantly higher body weight than those in the other groups despite that rats in group III had consumed 10% more energy due to sugar intake from the juice. Rats belonging to group II showed increased body weight, total cholesterol and triglycerides compared to the other groups (I and III). In addition, orange juice consumption increased insulin sensitivity when compared to group II. Expression of genes related to hepatic lipid metabolism was also analyzed in this research for peroxisome proliferator-activated receptor (*PPAR-α*), acyl-CoA oxidase (*AOX*), liver X receptor α (*LXR-α*), fatty acid synthase (*FAS*), hydroxymethylglutaryl-CoA reductase (*HMGCR*), and glycerol-3-phosphate acyltransferase 1 (*GPAT1*). PPAR-α is a key

transcription factor responsible for promoting lipolysis and lipid oxidation in different tissues. AOX promotes lipolysis, and LXR-α, FAS, and HMGCR are connected with lipogenesis in various tissues. GPAT1 is a key enzyme of the glycerolipid biosynthesis pathway. The gene expression analysis showed that Moro orange juice induced the expression of PPAR-α and AOX, and significantly decreased the gene expression levels of LXR-α, FAS, and HMG-CoA reductase. GPAT1 expression was markedly higher in group II, but its expression in group III was restored to the same levels of group I lean animals. Therefore, Moro orange juice has metabolic hepatoprotective effects by modulating the expression of the main genes involved in lipid homeostasis. The dietary administration of blood orange may be beneficial to prevent steatosis and could be considered as an approach for NAFLD prevention[44].

In summary, results in murine models encourage further clinical studies that are needed to address the beneficial effects of anthocyanin-rich diets for humans.

Importantly, the consumer's acceptance of beneficial phytochemicals in the diet has been widely recognized as an important factor in the orientation of new products in the health food market[48]. On the other hand, although people are becoming globally more aware of the benefits of nutrient density and functional foods, the manner that nutraceuticals are presented to society can influence and

have a long-lasting effect on the consumer's perceptions about these foods. As an iconic example, genetically modified (GM) foods have been available in the U.S. market since 1994[49], but a significant portion of the population remains apprehensive or contrary to the consumption of bioengineered crops[50-52]. In general, the consumers that care the most about the nutritional quality of their food are strongly against the consumption of GM products[53, 54]. In this context, although purple tomatoes demonstrated substantial protective effect against cancer progression[7], the fact that the anthocyanin biosynthesis was activated by a transgenic approach may condemn them to commercial failure. It is, therefore, essential that in addition to ensuring a high nutritional level, crop breeding strategies also accommodate consumers' cultural and ethical standards. Therefore, with the right approach, breeding food crops for high anthocyanin content may lead to an increase in market availability and dietary consumption of this phytochemical, potentially leading to substantial health benefits to the general population.

THE ANTHOCYANIN BIOSYNTHESIS PATHWAY

Anthocyanins are flavonoid pigments synthesized by the phenylpropanoid-acetate pathway (**Figure 2**). The entry metabolite is the aromatic amino acid,

phenylalanine (which is produced by the shikimate pathway). This precursor is first deaminated by phenylalanine ammonia lyase (PAL) in the cytoplasm, at the outer surface of the endoplasmic reticulum (ER) membrane[55, 56], to produce *trans*-cinnamic acid, which is then converted into *p*-coumaric acid (a.k.a. 4-coumaric acid or *trans*-*p*-hydroxycinnamic acid, pHCA) by cinnamate 4-hydroxylase (C4H, CYP73A cytochrome P450 monooxygenase). Alternatively, in some plant species, tyrosine can be converted to *p*-coumaric acid by tyrosine ammonia lyase (TAL) or even be used as a minor substrate by PAL[57, 58]. Thereon, *p*-coumaric acid, usually the most limiting intermediate in the phenylpropanoid pathway[59], is conjugated with coenzyme A to produce *p*-coumaroyl-CoA by 4-coumarate-CoA ligase (4CL). At this point, the phenylpropanoid pathway branches off to biosynthesis of coumarins, monolignols (lignans and lignin, aromatic volatiles – and in some species, salicylic acid) or polyketides (e.g., flavonoids, isoflavonoids, stilbenes and pyrones). The polyketide pathway advances with the condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to produce chalcone (a.k.a. naringenin chalcone or tetrahydroxychalcone) by chalcone synthase (CHS)[60]. Chalcone is converted by chalcone isomerase (CHI) to the flavanone naringenin, a central flavonoid intermediate. Thereon, flavanone 3-hydroxylase (F3H) converts naringenin into the flavononol dihydrokaempferol (a.k.a. DHK or aromadendrin), which is disputed by flavonoid 3’-hydroxylase (F3’H) to produce dihydroquercetin (a.k.a. taxifolin), and flavonoid 3’,5’-hydroxylase (F3’5’H) to

form dihydromyricetin (a.k.a. ampelopsin). Next, a set of enzymes with broad substrate specificity accepts flavononols to move the pathway forward. Dihydroflavonol 4-reductase (DFR) converts dihydrokaempferol (or the direct products of F3'H or F3'5'H enzymes) into leucoanthocyanidins, which are then converted into colored anthocyanidins (e.g., cyanidin, pelargonidin, delphinidin) by anthocyanidin synthase (ANS, a.k.a. leucocyanidin oxygenase: LDOX). These anthocyanidins can be further decorated by transferases, such as methyltransferases (OMT) and acetylases[61], and further processed by 3-*O*-glycosyltransferases (3GT, a.k.a. UDP-glucose:flavonoid-3-*O*-glycosyltransferase: UFGT) to produce anthocyanidin-3-*O*-glucosides, which are chemically stable, water soluble pigments. At last, anthocyanins are conjugated with glutathione by glutathione S-transferase (GST), which allows the escorting of these molecules for storage in the vacuole[62] with the assistance of ABC and MATE transporters localized at the tonoplast[63]. Alternatively, aggregates containing anthocyanins can be formed in the cytoplasm and are engulfed by the vacuole through tonoplast protrusions, thus forming anthocyanin vacuolar inclusions (AVI) in a process that resembles microautophagy[64].

Currently, more than 29 different molecule types have been identified only in Arabidopsis, including *cis* and *trans* isomers. Cyanidins are the predominant structure, while all other types are considered cyanidin derivatives carrying different modifications, such as glycosylation, acylation, and

methylation[65]. Furthermore, anthocyanin structures are classified numerically (A1, A2, … A19 and so forth), indicating different properties of solubility, light absorption, and distribution pattern in various parts of the plant[66]. For example, A11 molecules are most abundant in leaves[67], while A5 anthocyanins are most frequently found in roots[68].

It is relevant the fact that epidermal cells are more prone to accumulate anthocyanins than the parenchymal cells. However, this rule can be overturned in exceptional cases. In crops, there are many examples of variants with cyanic alternates: cauliflower[69], eggplant[70], tomato[13, 71] , grape[72, 73], citrus[43], pomegranate[74], apple[75], cherry[76], sweet cherry[77] ,bilberry[78], raspberry and blackberry[79, 80], peach[81], pear[82], potato[83], sweet potato[84], radish[85], carrot[86], rice[87, 88], and corn[89]. Despite the fact that genetic mechanisms have already been revealed to explain cyanic variations in some crops, the underlying basis for the general recalcitrance of parenchymal cells to synthesize and accumulate anthocyanins is still largely unknow.

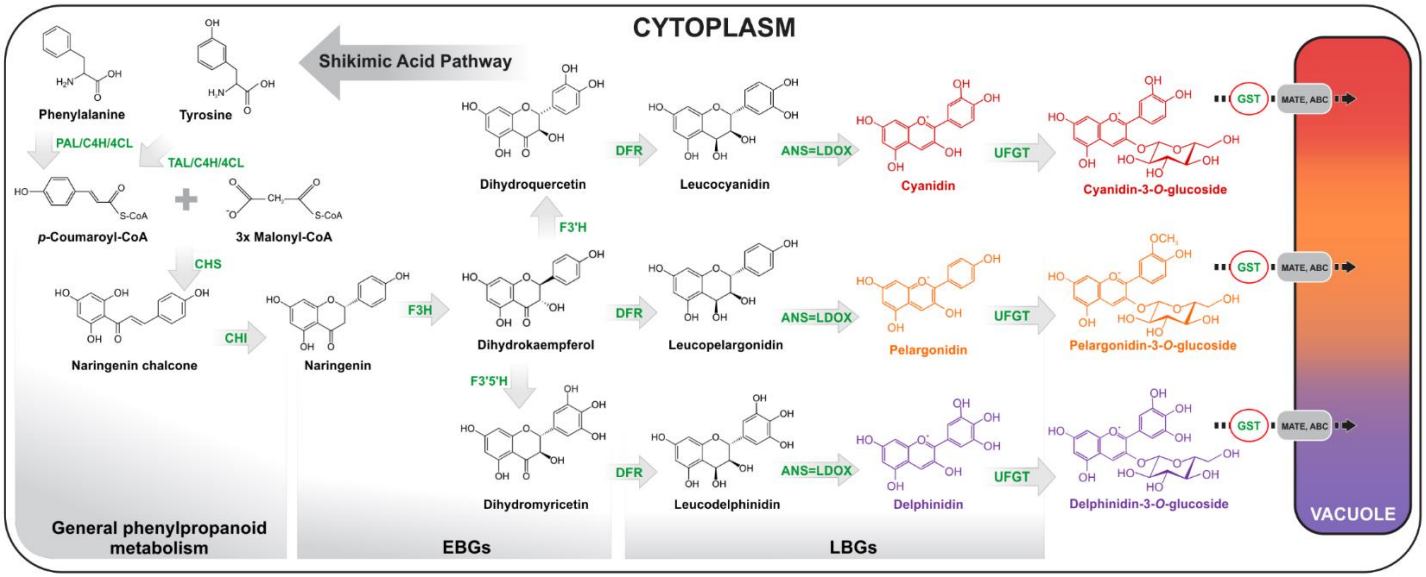

Figure 2. Anthocyanin biosynthesis pathway. Phenylalanine (and occasionally, tyrosine) enters the general phenylpropanoid pathway with PAL (or TAL) activity. CHS directs p-coumaroyl-CoA to the flavonoid pathway, and the route becomes committed to anthocyanin by the activity of F3H. The broad substrate specificity of DFR converts flavononols into leucoanthocyanidins, which are oxidized by ANS/LDOX, yielding anthocyanin pigments. Anthocyanins are further decorated with sugars and other moieties, increasing their stability and solubility. At last, they are conjugated with glutathione by GST and transported for storage into the vacuole via transporters in the tonoplast, or by the formation

of autophagic vacuolar inclusions. PAL: phenylalanine ammonia lyase; TAL: tyrosine ammonia lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumarate-CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; F3'5'H: flavonoid 3',5'-hydroxylase; DFR: dihydroflavonol 4-reductase; ANS: anthocyanidin synthase; LDOX: leucocyanidin oxygenase; UFGT: UDP-glucose:flavonoid-3-*O*-glycosyltransferase; GST: glutathione S-transferase; MATE: multi-antimicrobial extrusion protein; ABC: ATP-binding cassette transporter; EBGs: early biosynthesis genes; LBGs: late biosynthesis genes.

TRANSCRIPTIONAL REGULATION OF ANTHOCYANIN BIOSYNTHESIS IN THE ARABIDOPSIS MODEL

Although some branches of the phenolic pathway are restricted to certain species, such as phlobaphenes (in corn, kola nut, and Sequoia redwood) and isoflavones (in legumes), other compounds are broadly distributed in the plant kingdom, such as flavonols, proanthocyanidins, and anthocyanins[90-92].

An important regulatory mechanism of the flavonoid pathway is through the coordination of expression of structural genes, such as biosynthetic enzymes[93]. The structural genes of the flavonoid metabolism are usually conserved at the structural and functional levels, although small differences lead to the myriad of chemical diversity found even in closely related species. The expression of the so-called early flavonoid biosynthesis genes (EBGs: CHS, CHI, F3H, F3'H, and FLS) is modulated by MYB (MYB11, MYB12, MYB111) and bHLH transcription factors. On the other hand, the anthocyanin late biosynthesis genes (LBGs: DFR, ANS/LDOX, UFGT) and anthocyanin transporters embedded in the tonoplast (MATE, ABC) are regulated by a trio of transcription factors known as the MBW ternary complex (R2R3-MYB, bHLH, and WD40)[94].

The anthocyanin biosynthesis pathway and its genetic regulation mechanisms were characterized by a series of studies that used natural variants

with the loss of function of essential genes of the pathway by insertion transposable elements[95], and the epigenetic phenomena of paramutation and gene silencing[96, 97]. These analyses were largely facilitated by the easy visual phenotyping of mutants. These studies led to significant findings, such as the identification and characterization of plant transcriptions factors involved with the anthocyanin pathway[98, 99], not to mention the discovery of the co-suppression mechanism widely known today as RNA interference[100, 101].

The main R2R3-MYB transcription factors associated with the formation of the MBW complex and anthocyanin biosynthesis in Arabidopsis are PAP1/MYB75, PAP2/MYB90, MYB113, and MYB114[65]. Transgenic plants overexpressing *PAP1* (*PRODUCTION OF ANTHOCYANIN PIGMENTATION 1*) produce and accumulate high amounts of anthocyanins in roots, stems, leaves and flowers[67, 68, 102]. Consistently, *pap1* mutants are acyanic, confirming that this transcription factor is an essential positive regulator of the pathway in Arabidopsis. Studies have shown that in special environmental conditions or specific developmental stages of the plant, PAP2, MYB113, and MYB114 also activate the expression of essential genes, such as DFR and ANS[65], although to a minor extent when compared to PAP1[93, 100].

Regarding bHLH factors of the MBW complex, GLABRA3 (GL3), ENHANCER OF GLABRA 3 (EGL3) and TRANSPARENT TESTA 8 (TT8)

play redundant functions in modulating the proanthocyanidin and anthocyanin biosynthesis[103, 104]. Although both GL3 and EGL3 can stimulate F3'H expression, EGL3 is the predominant factor responsible for activating the LBGs DFR and ANS/LDOX[93]. For a long time, EGL3 was suggested to have the most prominent regulatory role during anthocyanin biosynthesis. However, a study conducted under low nitrogen conditions demonstrated that it is GL3 the most important regulatory factor in leaves during this condition[105]. These observations show that EGL3 and GL3 present functional specificities during development or stress. Finally, the TT8 protein is required for normal expression of two important LBGs, DIHYDROFLAVONOL 4-REDUCTASE (DFR) and the anthocyanidin reductase BANYULS (BAN) in Arabidopsis siliques and endothelial cells of the seed[106]. Moreover, transcriptome profiling of *pap1-d* mutant leaves overexpressing *PAP1-D* revealed that TT8 expression is strongly induced in seedlings[107].

Among the 269 genes in the Arabidopsis genome coding for proteins containing at least one WD-40 motif[108], to date, only TRANSPARENT TESTA GLABRA 1 (TTG1) has been characterized as a regulator of anthocyanin biosynthesis. TTG1 is expressed constitutively in all tissues and throughout plant development, with little response to changes in environmental conditions at the transcriptional level[109, 110]. Mutations in TTG1 resulted in numerous pleiotropic

effects, including defective trichome development, and deficient biosynthesis of proanthocyanidin and anthocyanins in vegetative tissues and the seed coat[95, 109].

Therefore, although the transcription factors mentioned above are demonstrably central regulators of anthocyanin production in Arabidopsis[93] and other species[111, 112]. The precise identification of which bHLH and MYB orthologs in other species form functional MBW complexes that activate the anthocyanin biosynthesis during the various stages of plant development and environmental conditions remains limited. Different (positive and negative) feedback mechanisms control the responses to environmental changes[67]. Such responses occur in a fast and precise way and are dependent on the coordinated action between activators and repressors. Cominelli and colleagues[109] demonstrated that under high light intensity situations, PAP1 is the first factor to be transcriptionally activated. Thus, together with EGL3 and TTG1, PAP1 is thought to compose the initial MBW complex that induces the pathway[109]. Possibly, this original complex activates the expression of TT8, which in turn competes with EGL3 for the complex. Importantly, TT8 expression is self-regulated by a positive feedback loop mechanism, leading to a robust activation of LBGs[113].

On the other hand, the transcriptional mitigation of MBW genes can be achieved by inhibitory MYBs. In Arabidopsis, the negative MYB regulators first discovered were MYB4 and MYB32, which are involved in lignin biosynthesis[114].

More recently, MYB7 and MYB4 were found to repress the flavonoid biosynthesis by negatively regulating DFR and UGT genes[115]. These factors form a distinct phylogenetic subclade of R2R3-MYB repressors. Additionally, the R3-MYB factors CAPRICE (CPC) and MYBL2 were identified as negative regulators specific to the anthocyanin pathway[116-118]. In addition to directly regulating the MBW complex by competing with positive MYB factors, MYBL2 is also capable of repressing the pathway indirectly by preventing the expression of positive regulators, such as TT8, PAP1, and PAP2[119]. Moreover, although TT8 self-promotes its own expression, it also activates the expression of its repressor, MYBL2, thus counterbalancing the activation mechanism (**Figure 3a**). In Arabidopsis, six other R3-MYB factors compose the CPC family: TRIPTYCHON (TRY), ENHANCER OF TRY AND CPC1 (ETC1), ETC2, ETC3/CPC-like MYB3 (CPL3), TRICHOMELESS1 (TCL1), and TCL2/CPC-like MYB4 (CPL4)[120-122]. Importantly, it does not go unnoticed that many genes that regulate the anthocyanin biosynthesis are also related to the development of trichomes and root hairs[95, 123].

High-light conditions rapidly inhibit MYBL2 expression, resulting in the activation of PAP1, PAP2, and GL3 expression, thus favoring a high anthocyanin accumulation[124]. Moreover, nitrogen deficiency conditions activated PAP1, PAP2, GL3, and MYB12 transcription and induced flavonoid accumulation[125]. On the other hand, low temperatures coupled with a high incidence of light enhanced

MYBL2 transcription, consequently inhibiting PAP1, TT8, TTG1, and EGL3 gene expression. MYBL2 expression also reduces anthocyanin biosynthesis in leaves, even in transgenic plants constitutively expressing PAP1[67]. Shi *et al.*[126] proposed that the quantitative competition between positive (PAP1, PAP2, MYB113, MYB114) and negative regulators (CPC, MYBL2), not to mention their affinities to the other components of the complex, determine the expression of the genes in the anthocyanin pathway (**Figure 3b**).

Finally, a recent study showed that among the twelve genes characterized in the proanthocyanidin biosynthesis pathway, only the LBGs DFR, ANS/LDOX, and BAN, along with TT19, TT12 and AHA10 are direct targets of the MBW complex[127]. The authors suggested that LBGs should include these latter genes as well. Congruently, the study also showed that the precise regulation of the MBW complex on LBGs genes occurs according to a particular fashion involving genes and tissues, and that not only one but different MBW complex arrangements participate in this mechanism[127]. Therefore, although the canonical MBW complex (TT2-TT8-TTG1) plays a central role in the modulation of the genes DFR, ANS/LDOX, BAN, TT19, TT12, and AHA10, the MYB5-TT8-TTG1 complex is active exclusively in the endothelium layer of the seed coat. This replacement induces the expression of DFR, ANS/LDOX, and TT12. Meanwhile, two other MBW complexes variants (TT2-EGL3-TTG1 and TT2-GL3-TTG1) are present in the chalaza to modulate the expression of DFR, ANS/LDOX, and BAN,

or DFR and AHA10, respectively[127]. These examples of alternative complexation in that different MYBs and bHLH factors participate in a spatiotemporal regulation of anthocyanin production illustrate the mechanistic sophistication by which plants ensure the degree of pigmentation in particular cells and tissues is appropriate under different environmental and developmental signals. However, much remains to be known regarding the genetic mechanisms governing the regulation of biosynthesis, transport, and accumulation of this pigment. For example, questions about the differences in genetic interactions that specifically regulate the anthocyanin pathway in different cell types, the molecular triggers of expression of basal regulatory genes, and the details of the vacuolar capture mechanisms of these polyphenols in the cell still need to be fully answered.

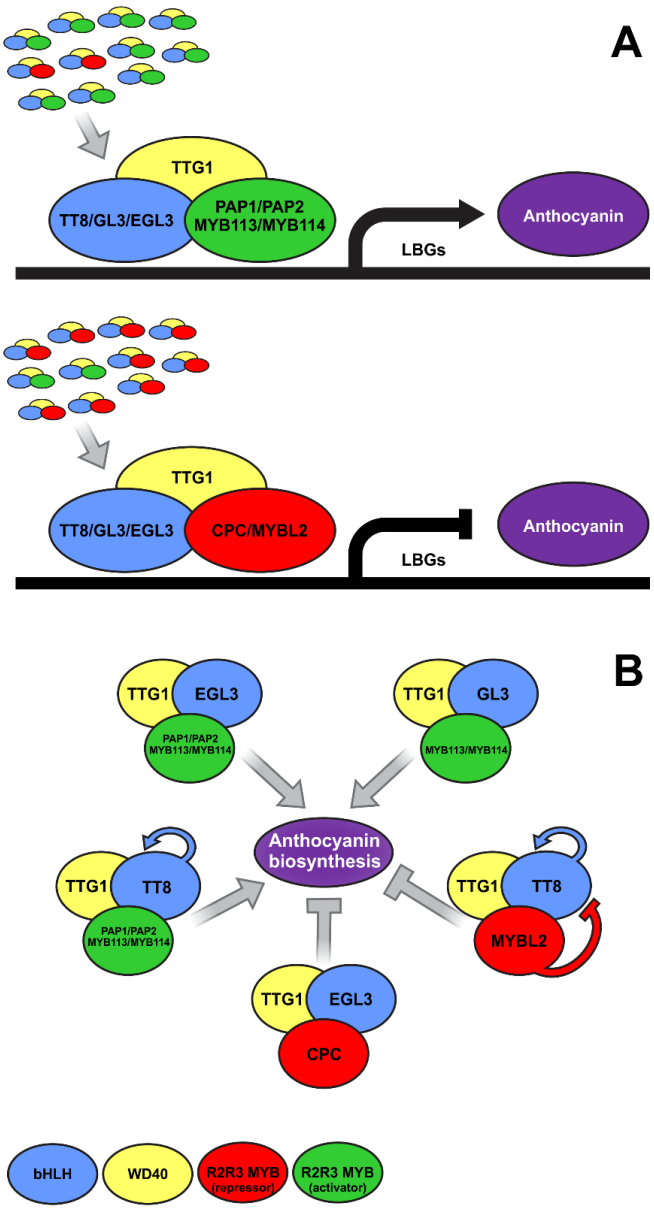

Figure 3. The MBW ternary complex formed by distinct transcription factors regulate the late biosynthesis genes (LBGs) of the anthocyanin pathway. **(A)** The ternary complex can act as an activator or repressor of anthocyanin biosynthesis

genes, depending on which MYB protein joins the complex. **(B)** For the formation of the MBW complex, the MYB protein plays a pivotal role in dictating the direction of the transcriptional control. Moreover, the bHLH factor TT8 shows positive feedback self-activation, which has not been reported for alternate bHLH factors, such as EGL3 and GL3. In turn, the repressor MYBL2 factor inhibits TT8 expression, thus allowing for a self-regulation mechanism. The alternative MYB repressor, CPC, has not been shown to possess such inhibitory activity on the expression of a bHLH factor. Regarding the WD40 factor of the complex, its expression is frequently not limiting for the transcriptional activation of the anthocyanin LBG.

GENETIC REGULATION OF ANTHOCYANIN BIOSYNTHESIS IN CULTIVATED PLANTS

Our knowledge on the regulatory mechanisms of anthocyanin biosynthesis in crops is not as vast as in Arabidopsis, but numerous studies have expanded our understanding in cultivated species. The identification of specific homologous genes playing similar roles to those identified in Arabidopsis is necessary, as well as the characterization of possible regulatory nuances in each species of interest. Furthermore, advancing our comprehension on how the anthocyanin pathway is regulated in specific tissues of fleshy fruits and other edible plant structures will better guide the efforts towards nutrient-dense breeding.

Fleshy fruits: the parenchymal recalcitrance of anthocyanin biosynthesis

Regarding the transcriptional regulation of anthocyanin synthesis, apple, strawberry, and grape are the best-studied fruit crops - as horticulturally defined, since botanically apple and strawberry are accessory fruits[128-130]. In apple, two different groups of red-fleshed fruit variants have been identified. In type-I apples, the red color occurs not only in the fruit (peel and flesh) but also in vegetative organs of the plant. On the other hand, the red pigmentation of type-II apples occurs exclusively in the fruit (peel and flesh as well), but not in vegetative structures. Besides the genetic difference responsible for pigmentation types of red-fleshed apples, they also show a different pattern of anthocyanin accumulation during fruit development. Type-I cultivars show intense pigmentation already during immature stages, reducing the color intensity of the fruit as ripening occurs. Meanwhile, type-II varieties bear fruits that are acyanic in early stages of development, and pigmentation is gained during ripening[131].

The type-I phenotype is caused by the expression of MdMYB10, a particular allele of MdMYBA, as well as MdMYB1, that together are responsible for inducing anthocyanin biosynthesis[75]. MdMYB1, MdMYBA, and MdMYB10 share 98% of nucleotide identity in the coding region, and possibly belong to the same locus or are paralogs derived from recent gene duplications. However, *cis*-

element rearrangements in the MdMYB10 promoter, such as the tandem addition of five identical 23-bp repeats, were sufficient to produce a self-regulatory locus to enhance transcription and subsequently cause constitutive anthocyanin accumulation, including in parenchymal cells of the fruit mesocarp[75]. When overexpressed in Arabidopsis, MdMYB10 strongly induced the expression of anthocyanin biosynthetic genes, and the pigment was produced in virtually all tissues of the plant[75]. The type-II pattern is independent of MdMYB10 expression. In varieties such as 'Pink Pearl' and 'JPP35', the red color of the fruit flesh is regulated by MdMYB110a[132], which is a dominant allele genetically linked to the S3-RNase locus responsible for self-incompatibility in 'Pink Pearl'[133, 134].

Much like in Arabidopsis, bHLH transcription factors (MdbHLH3 and MdbHLH33) also control the expression of anthocyanin biosynthesis genes in cyanic cells[75, 135]. Furthermore, despite the fact that the WD40 factor MdTTG1[136] is considered as an essential factor for the induction of polyphenol biosynthesis in apple, its ability to interact with MYB and bHLH proteins to form the MBW complex has not been fully confirmed yet. Apparently, MdTTG1 associates with the bHLH, but not with MYB proteins to regulate anthocyanin accumulation[137].

MdMYB10 orthologs have also been investigated in other fruit species of the Rosaceae family, such as pear, plum, cherry, peach, raspberry, and strawberry[137, 138]. In strawberry, FaMYB10 is not expressed until the fruit (i.e., the

fleshy receptacle) reaches its maximum size, when the expression correlates with ripening[129, 138]. Furthermore, FaMYB10 overexpression increased anthocyanin accumulation by 70% compared to non-transgenic wild strawberries[139]. Conversely, knockout mutants of this transcription factor failed to express anthocyanin biosynthetic genes (both, EBGs as LBGs), supporting the central positive role of FaMYB10 in anthocyanin metabolism in strawberry[140]. On the other hand, FaMYB1 was the first anthocyanin repressor characterized in fruit species[129, 141]. High FaMYB1 expression was observed in the ripen fruit, where it balances pigment accumulation in the late stages of fruit maturation[141]. Also, FaMYB1 expression is possibly the responsible for the white core of the fruit in many cultivars.

Given that anthocyanins are the pigments responsible for the red color of wine, there is an immense interest from this industry to better understand the control of the pathway in grape berries. So far, MYB factors have been confirmed to play crucial roles in the control of the phenylpropanoid pathway in the epicarp[70]. Anthocyanin synthesis in red cultivars is controlled by two MYB genes (VvMYBA1 and VvMYBA2), which specifically regulate the expression of the genes involved in the final steps of the pathway, such as UFGT, GST, OMT, and anthoMATE[69]. Congruently, some studies with red, black and white grape cultivars have shown that UFGT is the key enzyme that limits the accumulation of anthocyanin in several white grape varieties[142, 143]. While most red grapes show

non-pigmented flesh, a few genotypes (called *teinturier* varieties, such as 'Alicante Bouchet' and 'Salvador') develop cyanic flesh, and despite their poor vinification quality, they are used by the industry in blends to produce deep-colored wines.

The WD40 (VvWDR1 and VvWDR2) and bHLH (VvMYC1 and VvMYCA1) transcription factors were identified as possible components of the MBW complex in grapevine[69] . Their expression patterns are correlated with the VvMYBA1 and UF3GT transcriptional profiles as well as with the anthocyanin accumulation in the berry. Overexpression of VvWDR1 in Arabidopsis did activate anthocyanin biosynthesis, demonstrating the functional conservation of this genetic network since the last common ancestor that lived circa 115 million years ago[130, 144, 145]. Similar to TT8 in Arabidopsis, VvMYC1 regulates its own expression through a positive feedback mechanism. Studies showed that VvMYC1 interacts with all MYB factors analyzed (VvMYB5a/5b, VvMYBA1/A2, VvMYBPA1), and it is also able to induce the promoters of anthocyanin and proanthocyanidins biosynthetic genes in grapevine[146].

Regarding the structural genes in grape, Xie *et al.*[147] compared the expression of anthocyanin biosynthetic genes in the berry skin and pulp of *teinturier* and common grape varieties. The results showed that OMT, AM3, GST, F3'5'H, ANS/LDOX and MYBA1 genes were highly expressed in the pulp of

teinturier grapes while hardly detectable in the pulp of acyanic berries. The results strongly suggest that these are the main genes responsible for pigment accumulation in the flesh of *teinturier* berries[147].

Although some red-fleshed pear varieties do exist, there are no reports of research conducted on them yet. In Asian pear (*Pyrus pyrifolia*), a comparison between a red skin ('Aoguan') and an acyanic cultivar ('Mantianhong') showed that PyMYB10 and PyMYB10.1 are the primary MYB candidates to activate anthocyanin biosynthesis in the peel[82]. When assessing the epidermis of six different cultivars of European pear (*Pyrus communis*), Yang *et al.*[148] found no correlation between the expression of EBGs and anthocyanin content in the fruit, revealing that these enzymes were not limiting the pathway. Moreover, the expression of the LBGs ANS/LDOX and UFGT was shown to crucial to induce the pathway. Co-expression analysis revealed a strong association between the expression of MYB10 and bHLH33 and anthocyanin in cyanic fruit epidermis[148]. Surprisingly, however, was the negative correlation between the expression of a WD40 factor and anthocyanin accumulation. If confirmed, this protein could be functioning as a decoy element that competes with positive components of the ternary complex to halt the expression of structural genes[148].

Still in the Rosaceae family, the purple-leaf plum (cv. Ziyeli) accumulates anthocyanin throughout the plant, including the fruit flesh. Among the several

MYB factors evaluated in this species, the main gene affecting anthocyanin biosynthesis is PcMYB10.6, which was highly expressed in all cyanic organs[149]. In cherry (*Prunus avium*), PaMYB10.1-1 correlated with anthocyanin levels in the fruit, although PaMYB10.1-3, which is only moderately expressed in the fruit, was capable of inducing anthocyanin biosynthesis in tobacco leaf infiltration assay. Moreover, as for bHLH factors, PabHLH3 was shown to be a positive regulator, while PabHLH33 was a strong inhibitor of the biosynthesis. Like in other systems, PaWD40 expression is relatively constant and non-limiting, including in the fruit flesh[150].

The rich genetic diversity existing in pomegranate makes it possible to find genotypes bearing fruits with black, red, pink, green and white skin[74]. In a comparative study of skin fruit pigmentation and expression of the transcription factors PgAN1 (bHLH), PgAN2 (MYB), and PgWD40, Rouholamin *et al.*[74] demonstrated that PgWD40 and PgAN2 factors are the mainly responsible for cyanidin biosynthesis by controlling the expression of PgDFR and PgANS/LDOX. The PgWD40 expression pattern was considerably different among the genotypes studied, being induced up to 60-fold in varieties bearing black fruit compared with those with acyanic skin. The same trend was observed for the only structural gene analyzed in the study, PgDFR, for which virtually no expression was detected in green and white fruits. On the other hand, PgAN2 was continuously expressed in all four genotypes, but at a higher level in darker (black

and red) fruit skins. Therefore, in pomegranate, the MBW complex may be formed by PgAN1, PgAN2, and PgWD40, and that there is a positive correlation between PgAN2, PgWD40 and PgDFR expression levels and anthocyanin accumulation in the fruit skin[74]. In addition, Ben-Simhom *et al.* complemented Arabidopsis *ttg1-9* mutants by overexpressing PgWD40, with the restoration of anthocyanin accumulation in vegetative tissues, restitution of mucilage in the seed coat, as well as the growth of trichomes on stems and leaves[151]. These results confirm once again the high conservation of the genetic network centered on the MBW complex over the course of evolution.

In the genus Solanum, many species bear fleshy fruits that are naturally pigmented with anthocyanins and other flavonoids, such as black nightshade (*S. nigrum*), eggplant (*S. melongena*) and tomato (*S. lycopersicum*). Non-transgenic varieties of purple tomato (e.g., 'Indigo Rose' and 'Black Galaxy') were obtained by crosses of the cultivated species (*S. lycopersicum*) with compatible wild species, such as *S. lycopersicoides*, *S. chilense*, and *S. cheesmanii*, leading to successful breeding of plants producing fruits with higher anthocyanin content[152]. Moreover, the introgression of just three natural allelic variants from wild species - *Anthocyanin fruit* (*Aft*), *atroviolacium* (*atv*) and *high pigment 2* (*hp2*) – induced a dark purple color in the fruit's epicarp[13]. It is noteworthy the fact that in addition to anthocyanins, the triple allelic combination (*Aft*/*atv*/*hp2*) also induced high concentrations of ascorbate and lycopene, without neither loss in soluble solids

content in the fruit nor yield compared to near-isogenic, red-fruit plants[13]. This finding demonstrates that the trait 'high anthocyanin in fleshy fruit' does not compete for other relevant metabolic pathways or lower synthesis of other nutrients, but it can rather be improved along with the increase of other antioxidant types without any yield loss.

Comparative analyses of gene expression carried out in fruit tissues (skin and flesh) of cv. Micro-Tom (MT) along with near-isogenic introgressed mutants (*Aft*, *atv*, *hp2*) showed that the high anthocyanin accumulation observed in the skin of purple fruits of genotypes introgressed with the *Aft* locus is primarily regulated by a complex MBW composed of SlMYB114, SlbHLH150/SlTT8-like, and SlTTG1-like (our data). It is also plausible that the R3-MYB transcription factor SlTRY[153] acts as a repressor of the pathway. Moreover, a transgenic variety of purple tomato expressing two heterologous transcription factors from *Antirrhinum majus* (the bHLH *Delila* and the MYB *Rosea1*) reveals significant details. The high levels of anthocyanins in both the peel and flesh of the fruit, especially delphinidin-3-(*trans*-coumaroyl)-rutinoside-5-glucoside and petunidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside, shows that substrate is not limiting in the flesh. Therefore, the recalcitrance of parenchymal cells to accumulate anthocyanin is fundamentally genetic due to lack of expression of the MYB and bHLH elements of the MBW ternary factor, since the WD40 component is constitutively expressed in the tomato fruit (our data). Therefore, learning how

to turn on the expression these missing M and B elements in the fruit will lead to anthocyanin accumulation in parenchymal cells.

Eggplant also displays genetic variation with fruits showing peel that is entirely cyanic or acyanic, as well as bicolored in gradient or striping patterns, although the flesh remains virtually acyanic in all groups. By constitutively expressing just the transcription factor SmMYB1, Zhang *et al.*[70] produced plants accumulating anthocyanins in leaves, petals, stamens, as well as fruit tissues, including the flesh. Transcriptional analysis showed that most structural genes of the anthocyanin biosynthesis pathway were highly expressed in the transgenic lines compared to non-transformed controls, indicating the pivotal role of SmMYB1 in the pathway induction[70].

Finally, a distinct group of *Citrus sinensis* cultivars is collectively called blood oranges. They accumulate anthocyanins predominantly in the endocarp of the citrus fruit (hesperidium) encompasses the edible portion (pulp), whereas the exocarp (flavedo and albedo layers) remains golden, completely lacking anthocyanin. This phenotype is interesting given that in most species, anthocyanins accumulate preferentially in the exocarp. Compared with golden oranges, CHS, ANS/LDOX, and UFGT were differentially expressed and strongly correlated with anthocyanin levels in the pulp of blood oranges[154]. The first work published on anthocyanin genetic regulation in Citrus described CsMYB8 and

CsMYC2 as controlling the pathway, along with an R2R3-MYB factor called RUBY implicated in pulp anthocyanin levels[154]. While RUBY expression was only found in blood orange pulp, expression of the bHLH component (CsMYC2) of MBW complex was detected in both types, indicating that the major limitation to anthocyanin accumulation in the pulp of ordinary oranges is the absence of RUBY expression[155]. Following, the sequencing of the RUBY promoter region in cv. Moro (a blood variety) and Cadenera (a golden variety) revealed a 500-nucleotide insertion in the Moro promoter. The sequence of this insert resembles long terminal repeats (LTRs) of a *Copia* retrotransposons and can activate RUBY expression during cold stress. Indeed, low temperature is a major factor involved in pigmentation development in blood oranges during fruit ripening[43].

It is quite suggestive that in at least three independent instances (apple, blood orange, cauliflower – all belonging to different botanical families), an insertion in the promoter close to the transcription start site of a MYB gene associated with anthocyanin biosynthesis led to the ubiquitous accumulation of anthocyanin in diverse organs, including internal tissues. Therefore, it is possible that a repressive *cis*-element is blocking the constitutive activation of this gene. A closer analysis of the possibly conserved inhibitory element in this promoter region may unravel an efficient tool for breeding, especially with the emerging genome editing techniques.

In corollary, the general recalcitrance of anthocyanin accumulation in parenchymal cells does not seem to be due to substrate limitations. The MBW complex often lacks MYB and bHLH transcription factors in parenchymal tissues to induce the structural genes of the anthocyanin pathway, while the WD40 component is in most cases ubiquitously available. In two instances characterized in orange and apple, anthocyanin accumulation occurred in the fruit flesh due to promoter rearrangements in the region of MYB transcription factor (RUBY and MdMYB10, respectively) (**Table 1**). These lessons learned directly from crop species are essential to creating effective breeding strategies aiming to boost anthocyanin in fruit and other edible plant structures.

Table 1. Crop varieties with high-anthocyanin content in edible parts of the plant have been characterized genetically.

Crop species	Genotypes studied	Transcription factors involved	Tissues analyzed	Main findings	References
Apple (*Malus* spp.)	cv. Red Field OP and other red-fleshed genotypes vs. white-fleshed varieties	MdMYB10	fruit - epicarp, mesocarp; leaf	Appearance of 5x23-bp *cis*-element tandem repeat in gene promoter 275-bp upstream the MdMYB10 start codon led led to constitutive cyanic tissues (including red flesh in fruit) by autoregulatory transactivation.	75
Asian pear (*Pyrus pyrifolia*)	cv. Aoguan (red peel) vs. Mantianhong (white peel)	PyMYB10	Fruit skin, young leaf and flower	Overexpression of PyMYB10 in Arabidopsis induced anthocyanin biosynthesis in immature seeds; expression of PyMYB10 in Asian pear tissues correlates with anthocyanin accumulation.	82
Bilberry (*Vaccinium myrtillus*)	Wildly grown in Finland's boreal forest	VmTDR4 (MADS-box) and VmMYB2	Whole fruit	Silencing of VmTDR4 resulted in a reduction of anthocyanins and was correlated with expression supression of of CHS and MYB2 compared to the wild type.	78
Blackberry (*Rubus* spp.)	cv. Arapaho	RuMYB10	whole fruit – ripening stages	RuMYB10 expression is upregulated during ripening, when anthocyanin accumulates	79
Blood orange (*Citrus* x *sinensis*)	cv. Moro, Tarocco, Jingxian, and other blood varieties vs. blond oranges	Ruby (MYB)	Endocarp (flesh)	500-bp LTR of *Copia-like* insertion in promoter region (254-bp upstream the start codon) led to cold-induced expression of Ruby transcription factor; in the independent Jingxian blood orange variety, a 5-kb insertion occurs instead at 450-bp upstream the start codon.	43
Cabbage (*Brassica oleracea* var. *capitata*)	Red varieties (Royale, Cardinal, Cairo, Red Express) vs. green varieties	BrMYB2 and BrTT8 (bHLH)	Seedling, leaf	Expression of MYB and bHLH transcription factors correlated with expression of structural genes of the anthocyanin pathway as well as anthocyanin accumulation (in epidermal tissues); nutritional stresses induced anthocyanin accumulation.	159
Cauliflower (*Brassica oleracea* var. *botrytis*)	Graffiti (purple) vs. Stovepipe (white – progenitor genotype)	BobHLH1, and BoMYB2 (*Pr* locus)	inflorescence (curd), and major organs of the plant	Insertion of a *Harbinger* DNA transposon 373-bp upstream of the semi-dominant *Pr-D* mutant allele was found in cv. Graffiti as responsible for the purple phenotype; in young organs, anthocyanin accumulates in more internal tissues, rather than the epidermis.	69

Eggplant – gilo, bitter tomato (*Solanum aethiopicum* group *Gilo*)	Non-specified (material from the Chongqing Acad. of Ag Sciences)	SmMYB1	epicarp, and major organs of the plant	Overexpression of *SmMYB1* gene induces anthocyanin accumulation.	70
Grape (*Vitis vinifera*)	Several contrasting varieties, including *teinturier* cultivars (Petit Rouge, Gamay Fréaux, Morrastel-Bouschet)	VvMYBA1-2	epicarp (berry skin), root, leaf	The overexpression of *VlmybA1-2* from *V. labruscana* was sufficient to induce constitutive accumulation of anthocyanin by activating especially the final genes of the pathway (e.g., UFGT, OMT, GST and anthoMATE), inclusive in inner tissues.	72
Onion (*Allium cepa*)	California Red	MYB1	epidermis	Knockdown expression of MYB1 by RNAi suppressed anthocyanin accumulation in epidermal tissues; overexpression of MYB1 in white petal of snapdragon mutant (*rosea*^{dorsea}) complemented phenotype in epidermal cells, as well as in white garlic.	160
Peach (*Prunus persica*)	Redhaven; Roza, Fantasia	MYB10.1, MYB10.3, and bHLH3	epicarp, mesocarp, mesocarp around the stone	These three genes are clustered on a single locus (*Anther color*, *Ag*) in the genome and may be responsible for anthocyanin biosynthesis localized in the peel and flesh inner cortex near the endocarp (stone).	81
Potato (*Solanum tuberosum*)	Several, including acyanic, red skin/white flesh, and the purple skin/purple flesh (cv. Hei Meiren)	StAN1 (MYB), StMYBA1, StMYB113; StbHLH1, StJAF13 (bHLH),	tuber skin, tuber flesh, the vascular rings of a red-skin, white flesh variety	Multiple MYB transcription factors are capable of inducing the anthocyanin pathway; expression of both bHLH gens correlate with anthocyanin biosynthesis and are the limiting factors for activating the pathway.	83
Radish (*Raphanus sativus*)	Contrasting cultivars: acyanic (Seo Ho), green peel/pink flesh (Man Tang Hong), and red peel/white flesh (Hong Feng #1)	RsMYB	root (skin and flesh)	The study identified sequences of some regulatory and structural genes of the anthocyanin pathway and quantified anthocyanin in root tissues of contrasting varieties; a MYB cDNA was identified, but its role in the pathway was not demonstrated.	85
Strawberry (*Fragaria* x *ananassa*)	Sachinoka	FaMYB10	whole fruit (developmental time course)	Light and ABA promote anthocyanin biosynthesis by independently inducing FaMYB10; FaMYB10 overexpression promoted while RNAi decreased anthocyanin content in the fruit.	138
Strawberry (*Fragaria vesca* ssp. *vesca*)	Alpine	FvMYB10	whole fruit	FvMYB10 overexpression induced constitutive anthocyanin accumulation, while RNAi promoted an	139

				acyanic phenotype, confirming the key role of this gene on the pathway.	
Sweet cherry (*Prunus avium*)	Hong-Deng (red flesh) Tieton (red fruit) vs. 13-33 (yellow fruit)	PacMYBA	whole fruit (developmental time course) whole fruit (developmental time course)	PacMYBA overexpression in Arabidopsis led to development of pigmentation in seeds, and its knockdown via the virus-induced gene silencing (VIGS) inhibited anthocyanin biosynthesis to a certain extent in the fruit; ABA was shown to induce anthocyanin biosynthesis in the fruit by inducing PacMYBA, while cytokinin inhibits the pathway. Comparative transcriptome (RNA-Seq) of whole fruit in 4 different developmental stages led to identification of genes potentially involved in the anthocyanin pathway in the fruit, including a MYB10 (PacMYBA), other MYB, bHLH and WD40 transcription factors.	77 76
Sweet potato (*Ipomoea batatas*)	7 contrasting genotypes, including the purple-fleshed cultivars: Ayamurasak, Murasakimasari, Purple Sweet Lord	IbMYB1	Peel and flesh of tuberous roots, other organs	IbMYB1 is expressed primarily in the purple flesh of the tuberous root; IbMYB1 overexpression induced anthocyanin ubiquitous accumulation in sweet potato calli as well as throughout the Arabidopsis plant; detection of possible alternative splicing variants of IbMYB1, with unknown consequences; the ortholog IbMYB2 is not expressed in the purple flesh of the tuberous root, and slightly in special situations (during tuberous root development or in stored tuberous root), thus possibly playing only a secondary role.	84
Tomato (*Solanum lycopersicum*)	*Anthocyanin fruit* (*Aft*) and *atroviolacia* (*atv*) mutants, *Aft*/*atv* double mutant (purple peel), cv. Ailsa Craig	SlMYB113	fruit peel	Transcriptome analysis (*Affymetrix* microarray) correlated expression of SlMYB113 with anthocyanin biosynthesis and related genes in the fruit peel.	71

Beyond the fruit: anthocyanin accumulation in other edible plant structures

In addition to the fruit, other organs can often accumulate anthocyanins, such as leaves, stems, root, several floral structures, and roots. Most commonly, and much like the fruit, the epidermis displays most anthocyanin accumulation, while the parenchymal cells of the cortex show recalcitrance to the pathway. This imposes a severe obstacle to breeding vegetables accumulating high amounts of anthocyanin in edible structures.

Among the many genotypes of potato (*Solanum tuberosum*), some develop completely non-pigmented tubers, while some show red skin and acyanic starchy parenchyma (flesh) and others have dark purple tubers that accumulate anthocyanins in both, peel and flesh. The epidermis of purple potatoes usually stores anthocyanins at much higher levels than the flesh, where the expression of structural genes of the pathway is strongly reduced[83, 156]. In Solanaceae, the bHLH transcription factors StJAF13 and StAN1 are responsible for hierarchically controlling this trait, with the former activating the expression of the latter, which then turns on structural genes[157]. Heterologous expression of StAN1 activated the expression of CHI, F3'H, F3H, and ANS/LDOX in tobacco flowers. Furthermore, NtAN1 and NtJAF13 silencing via RNAi produced plants with white and pale pink flowers, respectively[157].

In purple cultivars of leafy structures as evolutionarily divergent as onions and cabbage, it is interesting to note the distinct anthocyanin accumulation in the epidermal cells. The onion bulb is formed by a compact cluster of superimposed modified leaves (tunic) on a short basal stem (plate)[158]. Comparably, the cabbage head is also formed by overlapping, compacted leaves[159]. Anthocyanin accumulation in the epidermis of purple varieties in both inner and outer layers of the head reveals that, at least in some species, the incidence and quality of light are not always decisive factors for the differential anthocyanin accumulation between external and internal tissues of the plant, where light is restricted. Therefore, although light often is a major factor in promoting anthocyanin accumulation in plant tissues, there are cases that this is not the case. This fact is especially evident in underground structures with cyanic varieties, such as onion, potato, sweet potato, and carrot.

The R2R3-MYB transcription factor MYB1 is the main anthocyanin regulator in purple onions (cv. California Red)[160]. Anthocyanin biosynthesis was increased in tissues overexpressing MYB1, whereas the opposite was observed with RNAi lines. In addition, ectopic red pigmentation was found in garlic plants when MYB1 was overexpressed together with a bHLH transcription factor (Zm-Lc), but not when expressed alone.

Comparative analysis of expression of structural genes of the anthocyanin pathway between purple and green varieties of cabbage (*Brassica oleracea*), showed that both EBGs (CHS, F3H, and F3'H) and LBGs (DFR, ANS/LDOX, and GST) are positively regulated in purple genotypes. Although no drastic change was observed for BoPAL1 and CHI expression levels between the cultivars, the expression of a later gene of the pathway, ANS/LDOX, was almost 100-fold higher in purple than green varieties[159]. Regarding the regulatory genes, both BoMYB2 and BoTT8 (bHLH) were highly expressed in the leaf epidermis of purple varieties, whereas BoMYB3 was negatively correlated with anthocyanin content. Therefore, the respective positive and negative feedback mechanisms of BoMYB2 and BoMYB3 that regulate the expression of one another is evolutionarily conserved in cabbage[159].

In Chinese cabbage (*Brassica rapa* ssp. *pekinensis*), purple varieties also accumulate anthocyanins, particularly between the first and third cell layers of cells of the epidermis in edible tissues (head leaves) and reproductive organs. A regulation mechanism very similar to that of cabbage was observed in this species, in which the induction of the structural genes CHS, F3'H, DFR, ANS/LDOX, UFGTs, and GSTs is positively regulated mainly by BrMYB2 (orthologous to PAP2/MYB113 in Arabidopsis) and BrTT8[161]. Likewise, in several other cruciferous vegetables, MYB2 and TT8 factors were characterized as the primary activators of the anthocyanin pathway, including purple bok choy (*B. rapa* var.

chinensis)[162], kale (*B. oleracea* var. *acephala* f. *tricolor*[163], and cauliflower (*B. oleracea* var. *botrytis*)[164]. Remarkably, the purple phenotype in cauliflower is caused by a 695-bp *Harbinger* DNA transposon insertion in the BoMYB2 promoter[69]. This mutation activates the tissue-specific expression of BoMYB2, triggering the high accumulation of anthocyanins in the inflorescence, seeds, and young vegetative tissues. Thereupon, the accumulation pattern of anthocyanins in purple cauliflower (cv. Graffiti) is quite distinctive because it is not restricted to the outer cell layers, but also occurs in the central region of the inflorescence meristem, near the vascular bundles[164]. Thus, the purple cauliflower displays substantially high levels of anthocyanins although the biosynthesis regulation mechanism underlying this pattern remains unknown.

Purple and "black" varieties of carrot (*Daucus carota* ssp. *sativus* var. *atrorubens*) have existed for over 3,000 years, and are older than the orange genotypes[165]. Cyanic cultivars develop pigmented external (and sometimes internal) root tissues, in which the anthocyanin pathway is expressed in a light-independent fashion[86]. A transcriptional analysis comparing purple and orange carrots showed a high correlation between anthocyanin accumulation and expression of the structural genes PAL3/PAL4, CA4H1, 4CL1, CHS1, F3H1, F3'H1, DFR1, LDOX1/LDOX2[86]. However, so far no work has been reported on transcription factors regulating the anthocyanin biosynthesis in cyanic carrots.

At last, in tuberous roots of sweet potato (*Ipomoea batatas*), the pigmentation of the skin and flesh can vary substantially. Purple, red, pink, orange, yellow and white cultivars are available. Purple varieties can develop entirely pigmented roots[84]. The best-characterized transcription factor involved in this trait is IbMYB1, which induces anthocyanin biosynthesis exclusively in the tuber cortex, with no activity in non-tuberous roots, stem, leaf or floral tissues[84]. Its overexpression is sufficient to activate anthocyanin biosynthesis even in orange cultivars[166]. A comparative study between a purple cultivar (Ningzishu 1, N1) and its acyanic alternate (Mutant of Ningzishu 1, MN1) showed that IbMYB1 transcription was much higher in the purple genotype, and induced the late biosynthetic genes CHS, CHI, DFR, F3H, ANS/LDOX and UFGT[167]. Other transcription factors differentially expressed between the two genotypes remain to be characterized, including 17 bHLH factors[167].

OUTLOOK AND PERSPECTIVES

Phytonutrients have been largely overlooked in dietary guidelines. Despite the healthy skepticism about the benefits of dietary anthocyanins to human health due to the lack of direct evidence, well-designed studies on mammal models are pointing towards potential significant benefits against several maladies that

plague the human society. Therefore, until proven otherwise, diet recommendations should start suggesting the inclusion of anthocyanins through colored vegetable, given the ease of implementation, since cyanic foods are already available in the market, as well as the safety and low costs associated with this recommendation.

The regulation and metabolic pathway of anthocyanins in plants are, to a certain extent, well known at the cellular, physiological and genetic levels. However, a better understanding of the initial transcriptional triggers of activators and repressive transcription factors in parenchymal cells is much needed to enable the generation of breeding tools aiming to produce fruits and vegetables with internal tissues boosted with anthocyanins.

ACKNOWLEDGEMENTS

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES) from the Brazilian Ministry of Education for the full scholarship provided to A.L.S. and S.C.S. Luiz Carlos Rodrigues (UFES, Federal University of Espírito Santo) is recognized for assisting with figure edition. Scientific Article No. XXXX of the West Virginia Agricultural and Forestry Experiment Station, Morgantown.

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ARTIGO 2: CULTURA DO TOMATEIRO E CARACTERÍSTICAS DO MUTANTE TRIPLO *Aft/atv/hp2*

A ser submetido para REVISTA HORTICULTURA BRASILEIRA

CULTURA DO TOMATEIRO E CARACTERÍSTICAS DO MUTANTE TRIPLO *Aft/atv/hp2*

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RESUMO

O tomateiro é cultivado no mundo todo sendo o Brasil ocupante do oitavo lugar. O tomate possui importantes valores nutricionais como a presença de vitaminas A, B1, B2, B3, C, manganês, zinco, potássio, sódio, cálcio, ferro, fósforo, e outros compostos como carotenóides e flavonóides. Dentre os flavonoides, a antocianina tem se destacado nos vegetais, sendo seus benefícios à saúde já amplamente relatados. Infelizmente, frutos da espécie *Solanum lycopersicum* normalmente não produzem altos níveis de antocianinas. Entretanto, recentemente, foi produzido um mutante triplo de tomateiro *(Aft/atv/hp2)*, onde seu fruto é roxo devido a capacidade de acumular antocianina em seu epicarpo. Ademais, frutos *Aft/atv/hp2* apresentam características nutricionais interessantes como aumento dos níveis de carotenóides (licopeno) e vitamina C (ascorbato). Frutos *Aft/atv/hp2* acumulam antocianina desde o início do desenvolvimento do fruto, diferente da grande maioria dos frutos que acumulam esse pigmento durante sua maturação. Neste trabalho nós relatamos algumas características atribuídas ao genótipo mutante triplo *Aft/atv/hp2*. Observamos que a luz é o principal fator regulador da ativação da biossíntese de antocianina em frutos *Aft/atv/hp2*, ademais, constatamos que o acúmulo de antocianina nesses frutos se inicia durante a senescência floral e acontece especificamente no epicarpo. Apesar do genótipo mutante apresentar um retardo em seu desenvolvimento em relação a plantas selvagens, os frutos de ambos genótipos não apresentam diferença de tamanho quando maduros. Além disso, plantas mutantes aparentam possuir maior tolerância ao ataque de trips quando comparada a plantas selvagens. Enfim, esses relatos inéditos sobre a característica do recente genótipo mutante *Aft/atv/hp2* abrem portas para pesquisas futuras.

Palavras-chave: *Solanum lycopersicum*; Antocininas; Flavonóides; Tomte roxo.

ABSTRACT

The tomato is cultivated worldwide and Brazil occupies the eighth place. The tomato has important nutritional values such as the presence of vitamins A, B1, B2, B3, C, manganese, zinc, potassium, sodium, calcium, iron, phosphorus, and other compounds such as carotenoids and flavonoids. Among the flavonoids, anthocyanin has been prominent in vegetables, and its health benefits have already been widely reported. Unfortunately, fruits of the *Solanum lycopersicum* species do not normally produce high levels of anthocyanins. However, a triple tomato mutant (*Aft/atv/hp2*) has been produced recently, where its fruit is purple due to the ability to accumulate anthocyanin in its epicarp. In addition, *Aft/atv/hp2* fruits present interesting nutritional characteristics such as increased levels of carotenoids (lycopene) and vitamin C (ascorbate). *Aft/atv/hp2* fruits accumulate anthocyanin from the beginning of fruit development, unlike the great majority of the fruits that accumulate this pigment during its maturation. In this work we observed some characteristics attributed to the triple *Aft/atv/hp2* mutant genotype. We observed that light is the main regulating factor of the activation of anthocyanin biosynthesis in *Aft/atv/hp2* fruits, in addition, we found that the accumulation of anthocyanin in these fruits starts during floral senescence and occurs specifically in the epicarp. Although the mutant genotype presents a delay in its development in relation to wild plants, the fruits of both genotypes do not present difference in size when mature. In addition, mutant plants may have a greater tolerance against thrip when compared to wild plants. Finally, these unpublished reports on the characteristic of the recent *Aft/atv/hp2* mutant genotype open the door to future research.

Keywords: *Solanum lycopersicum*; Anthocyanin; Flavonoids; Purple tomato.

INTRODUÇÃO

O tomateiro é cultivado no mundo todo, onde China, Estados Unidos da América e Índia são os principais produtores, sendo o Brasil ocupante do oitavo lugar neste ranque. O tomateiro é uma das hortaliças mais produzidas e consumidas no Brasil, ocupando o segundo lugar na produção. Em 2016 a cultura do tomateiro ocupou a área de 56 034 ha^{-1}, com produção total de 3.626.408 toneladas com rendimento médio de 64,7 t ha^{-1}. No Brasil, os maiores estados produtores são Goiás com 817,8 mil toneladas, São Paulo com 753,3 mil toneladas, seguido de Minas Gerais com 739,5mil toneladas (IBGE, 2016).

O tomateiro é originário da América do Sul, mais precisamente na região da Cordilheira dos Andes. O antepassado mais provável do tomate cultivado é um pequeno tomate silvestre *L. esculentum* Mill var. *cerasiforme*, que cresce facilmente nas regiões tropicais e subtropicais da América e da Europa. Esta espécie dicotiledônea pertencente à família das Solanaceae planta perene de porte arbustivo que se cultiva como anual, possui caule flexível e incapaz de se suportar na posição vertical devido ao peso da parte vegetativa e dos frutos. O desenvolvimento inicial é relativamente lento quando comparado com desenvolvimento vegetativo acelerado da planta que ocorre simultaneamente com a floração e a frutificação. O ciclo cultural varia de 4 a 7 meses, da semeadura até a produção de novas sementes, incluído o período de colheita que pode variar de 1 a 3 meses. As flores se agrupam em cachos, são hermafroditas o que favorece a autopolinização, sendo o tomateiro domesticado uma espécie autógama. Entretanto, a fecundação cruzada pode ocorrer através de insetos (zoocoria). Os frutos são bagas carnosas, suculentas, variando em aspecto, tamanho e peso – de 10 até 500 g, dependendo da cultivar. Também varia o formato, podendo ser globular, cilíndrico, piriforme ou oblongo. O número de lóculos é variável de 2

(biloculares) até 10 (pluriloculares). A maioria das cultivares produz frutos de coloração vermelha bem viva, resultante da combinação da coloração rosada da polpa com a película amarela. A coloração do fruto é determinada por pigmentos como: licopeno de cor vermelha, caroteno de coloração amarela e antocianina de coloração roxa. Essas colorações de frutos são dependentes de temperatura, calor e luz respectivamente (FILGUEIRA, 2003; DÍEZ & NUEZ, 2008; LØVDAL et al., 2010).

As características nutricionais dos frutos de tomate podem variar de acordo com as condições ecofisiológicas e agroecológicas. De acordo com Filgueira, 2003 (FILGUEIRA, 2003), em 100 g de tomates frescos tem-se: 1% de fibra, 95,20% de água, 60 µg de vitamina A (retinol), 80 µg de vitamina B1 (tiamina), 113 µg de vitamina B2 (riboflavina), 0,450 µg de vitamina B3 (niacina), 15- 34,3 mg de vitamina C (ácido ascórbico), 20 mg de cobre, 14 mg de enxofre, 13 mg de magnésio, 0,1 mg de manganês, 0,2 mg de zinco, 209,4 mg de potássio, 42,0 mg de sódio, 9 mg de cálcio, 1,67 mg de ferro e 43 mg de fósforo, além de carotenoides e flavonóides (GEORGIEVA et al., 2014). Entretanto esses valores nutricionais podem variar dependendo da cultivar analisada.

Normalmente as variedades de tomateiro não acumulam antocianina em seus frutos. Antocianinas são pigmentos oriundos do metabolismo secundário dos vegetais e são responsáveis pela coloração vermelha, azul, violeta e rosa em tecidos vegetais como frutos, além disso, as antocianinas agregam valor nutricional ao fruto conferindo diversos benefícios à saúde dos consumidores (BUTELLI et al., 2008; TOUFEKTSIAN et al., 2008; KAWASAKI et al., 2014; CHAREPALLI et al., 2015; SU et al., 2016). Sendo o tomate uma das hortaliças mais consumidas em todo mundo (CARVALHO & PAGLIUCA, 2007), é de grande valia a obtenção de frutos de tomate com acúmulo de antocianinas.

Entretanto, frutos da espécie *Solanum lycopersicum* normalmente não produzem altos níveis de antocianinas, porém, em algumas espécies selvagens de tomateiro como *Solanum lycopersicoides, S.* cheesemanii e *S. chilense* podemos observar frutos consideravelmente pigmentados. Além dessas espécies selvagens, o mutante triplo (*Aft/atv/hp2)* possui a capacidade de acumular antocianina em seu epicarpo. Ademais, frutos *Aft/atv/hp2* apresentam características nutricionais interessantes como aumento dos níveis de carotenóides (licopeno) e vitamina C (ascorbato) (SESTARI et al., 2014).

A grande maioria dos frutos que acumulam antocianina, como por exemplo uva, amora, jabuticaba e berinjela começam a acumular antocianina durante o estádio de maturação do fruto, com isso, o fruto vai modificando sua coloração no decorrer da maturação. Fato diferente acontece com o genótipo mutante *Aft/atv/hp2*, onde os frutos são roxos por acumularem antocianina desde o início de seu desenvolvimento. O conhecimento ainda é pouco sobre as características do mutante *Aft/atv/hp2*, neste sentido é de primordial importância relatos sobre suas características afim de abrir caminhos para mais pesquisas sobre esse genótipo.

Nossos objetivos com este trabalho foram: i) definir o padrão de acúmulo de antocianinas durante o desenvolvimento do fruto *Aft/atv/hp2*; ii) verificar a influência da luz no acúmulo de antocianina nos frutos *Aft/atv/hp2* e iii) documentar características preliminares mais distintivas de plantas e frutos *Aft/atv/hp2* observadas em relação ao genótipo controle.

MATERIAL E MÉTODOS

IDENTIFICAÇÃO DA FASE INICIAL DE ACÚMULO DE ANTOCIANINA EM FRUTOS A*ft/atv/hp2*

Plantas selvagens (Micro-Tom) e plantas mutantes (MT- *Aft/atv/hp2),* foram cultivadas em mesmas condições em casa de vegetação sob foto período de 12 horas. Os botões florais foram coletados durante seu desenvolvimento e as flores foram coletadas em 3 momentos, imaturas, maturas e no momento da senescência floral, tais estádios foram identificados segundo ATHERTON & RUDICH, 2012 (ATHERTON & RUDICH, 2012). Os órgãos coletados foram observados em lupa (Nikon SMZ-U ZOOM 1:10) e fotografados (Câmera Nikon DS-Ri1 Câmera Nikon DS-Ri1). Os botões florais foram fotografados inteiros e após cortados transversalmente. As flores foram fotografadas com pétalas e sem elas. Todas as fotos foram analisadas no software NIS-Element BR 3.20.01 (build 685).

VERIFICAÇÃO DA INFLUÊNCIA DA LUZ NO ACÚMULO DE ANTOCIANINA EM FRUTOS *Aft/atv/hp2*

Para verificar a influência da luz no acúmulo de antocianina nos frutos de plantas *Aft/atv/hp2*, flores foram cobertas com papel alumínio (40 dias após a germinação). O papel alumínio foi retirado das plantas em duas épocas, após 30 e 60 dias após a cobertura com papel alumínio.

CORTES HISTOLÓGICOS DO FRUTO *Aft/atv/hp2*

Cortes histológicos foram realizados em frutos maduros *Aft/atv/hp2,* afim de observar as camadas de células pigmentadas. Foram realizados cortes transversais à mão livre utilizando lâminas de aço com borda dupla. Logo em seguida os cortes foram visualizados em microscópio (NiKon ECLIPSE E600) e fotografadas (Câmera Nikon DS-Ri1 Câmera Nikon DS-Ri1).

CARACTERÍSTICAS DO DESENVOLVIMENTO DE PLANTAS *Aft/atv/hp2*

Outras características relatadas foram observadas e fotografadas durante o desenvolvimento das plantas.

RESULTADOS E DISCUSSÃO

Por meio do acompanhamento desde o desenvolvimento dos botões florais pudemos identificar o real estádio onde acontece o início do acúmulo de antocianina nos tomates mutantes (Figura 1). Podemos observar que genótipos selvagens no mesmo estádio não possui a capacidade de acumular antocianina (Figura 2).

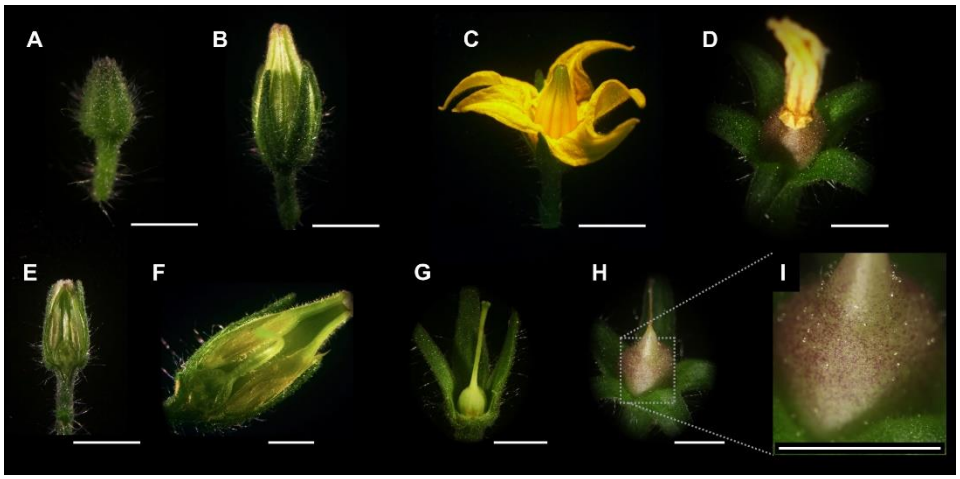

Figura 1. Botões florais, flores e fruto de plantas *Aft/atv/hp2*. Botão floral em desenvolvimento (A); Flor imatura (B). Flor em antese (C); Senescência floral (D); Corte transversal do botão floral em desenvolvimento (E); Corte transversal da flor imatura (F); Flor em antese sem pétalas (G); Fruto no início do desenvolvimento na senescência floral (H); Zoom do fruto no início de desenvolvimento (I).

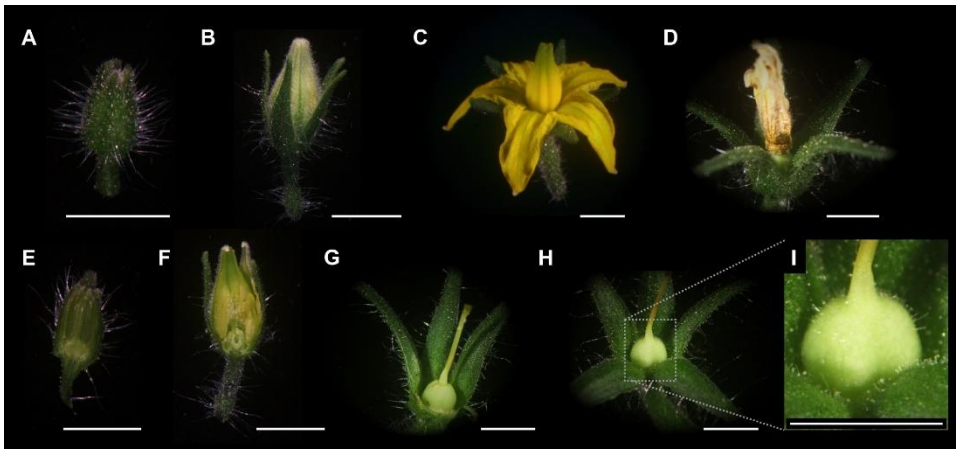

Figura 2. Botões florais, flor e fruto de plantas *Aft/atv/hp2*. Botão floral em desenvolvimento (A); Flor imatura (B). Flor em antese (C); Senescência floral (D); Corte transversal do botão floral em desenvolvimento (E); Corte transversal da flor imatura (F); Flor em antese sem pétalas (G); Fruto no início do desenvolvimento na senescência floral (H); Zoom do fruto no início de desenvolvimento (I).

O acúmulo de antocianina em tomates *Aft/atv/hp2* inicia-se durante a senescência floral, devido a incidência de luz nos frutos. Uma vez que,

anteriormente os frutos estavam protegidos pelas pétalas, com a senescência floral os frutos começam a receber estímulo luminoso e consequentemente ativa a biossíntese de antocianina. Esse resultado condiz com a literatura, onde já é relatado que os locus *Aft, atv* e *hp2* estão relacionados com a sensibilidade à luz e consequentemente ao acúmulo de antocianina em tomate (KENDRICK et al., 1997; MES et al., 2008; SAPIR et al., 2008).

Como observado na Figura 1, no genótipo *Aft/atv/hp2*, os frutos são roxos por acumularem antocianina desde o início do desenvolvimento do fruto, ou seja, mesmo estando em seu estádio imaturo (coloração interna verde), seu epicarpo já apresenta intensa coloração roxa (Figura 3).

Figura 3. Tomates *Aft/atv/hp2* imaturos. Tomates com 15 dias após antese (A); Frutos com 30 dias após antese (B).

Inicialmente este fato gerou uma pequena dúvida, pois seria difícil a identificação dos estádios de maturação dos frutos, já que desde o início do seu desenvolvimento ele já possui coloração roxa. Essa dificuldade foi sanada observando a coloração do fruto logo abaixo da sépala. Uma vez que a luz estimula o acúmulo de antocianina nos frutos, a parte do fruto logo abaixo da

sépala não é estimulada pela luz apresentando coloração verde quando imaturo ou vermelho quando maduro (Figura 4).

Figura 4. Tomates *Aft/atv/hp2* maduros (50 dias pós antese). Fruto com sépala (esquerda); fruto sem sépala (direita). No fruto da direita podemos observar coloração vermelha onde se encontrava a sépala.

Quando as flores foram cobertas com papel alumínio, os frutos ficaram protegidos da incidência de luz e não acumulam antocianina. Quando o papel alumínio foi retirado durante a fase inicial de desenvolvimento dos frutos (30 dias após ser coberto), observamos que os frutos não acumularam antocianina e apresentaram coloração branca, entretanto, após 24 horas, observamos que esses frutos começaram a acumular antocianina. Contrariamente, quando o papel alumínio foi retirado após a maturação dos frutos (60 dias após ser coberto), observamos que os frutos apresentavam coloração vermelha e, mesmo após 24 horas recebendo estímulo luminoso, aparentemente, os frutos não acumularam antocianina. Esses resultados nos levam a inferir que os genes chave da rota de biossíntese de antocianina em frutos *Aft/atv/hp2* somente são ativados quando o estímulo luminoso acontece no estádio inicial de desenvolvimento do fruto, após sua maturação, mesmo recebendo estímulo luminoso, não ocorre a ativação da rota de biossíntese de antocianina nesse genótipo (Figura 5).

Figura 5. Plantas cobertas com papel alumínio 40 dias pós germinação (A); plantas sem papel alumínio após 30 dias coberto (B); plantas sem papel alumínio após 30 dias coberto ao lado de plantas que não foram cobertas (C); plantas sem papel alumínio (após 30 dias + 24 horas) ao lado de plantas que não foram cobertas (D); plantas sem papel alumínio após 60 dias coberto (E); plantas sem papel alumínio após 60 dias coberto + 24 horas (F).

Apesar de não terem sido realizadas análises quantitativas de crescimento e desenvolvimento dos diferentes genótipos (Micro-Tom e mutante MT-*Aft/atv/hp2*), durante o cultivo dos tomateiros sob condições homogêneas em casa de vegetação, foi observado (visualmente), que o tomateiro mutante possui um retardamento de aproximadamente 10 dias em relação ao tomateiro selvagem

(Figura 6). Pode-se observar que aos 30 dias pós germinação as plantas selvagens já possuem frutos, enquanto plantas mutantes ainda estão no início do florescimento. Devido ao retardamento no desenvolvimento das plantas mutante, os frutos desse genótipo consequentemente têm seu estádio de maturação retardado comparado ao genótipo selvagem. Entretanto, frutos de plantas selvagens e mutantes quando maduros não diferem no tamanho, apenas na pigmentação (Figura 7).

Figura 6. Desenvolvimento das plantas 30 dias pós germinação. Plantas selvagens à direita; plantas mutantes à esquerda.

Figura 7. Fruto mutante (*Aft/atv/hp2*) à esquerda; fruto selvagem (Micro-Tom) à direita.

Além disso, foi observado uma certa tolerância do tomateiro mutante à trips (Thysanoptera). Infelizmente, durante a condução da pesquisa, mesmo em casa de vegetação, houve uma incidência de trips. Ambas cultivares se encontravam nas mesmas condições, sobre a mesma bancada dentro da casa de vegetação, devido ao ataque perdemos todas nossas plantas da cultivar selvagem, contrariamente, as plantas mutantes conseguiram sobreviver. Neste sentido, podemos inferir que, provavelmente as plantas mutantes possuem a capacidade de acumular algum composto em suas folhas oriundo do metabolismo secundário, capaz de torná-la mais tolerante ao ataque da trips. Diversos artigos já relacionaram o acúmulo compostos secundários em plantas com a tolerância a insetos e patógenos (LATTANZIO et al., 2006; BARBEHENN & CONSTABEL, 2011; MITHÖFER & BOLAND, 2012; WAR et al., 2012; SÁNCHEZ et al., 2016; SUN et al., 2017), inclusive antocianina (SIVANKALYANI et al., 2016; WEST, 2016). Entretanto são necessários mais estudos afim de comprovar este real efeito do genótipo (*Aft/atv/hp2).*

No corte histológico do fruto maduro *Aft/atv/hp2* podemos observar que o acúmulo de antocianina parece acontecer não nas células da epiderme (primeira camada de células), mas sim nas camadas de células abaixo dela (Figura 8). O acúmulo de antocianina acontece especificamente nas células do epicarpo, sendo inexistente o acúmulo do pigmento em células do mesocarpo. Esse padrão de acúmulo de antocianina também pode ser observado em outros alimentos como por exemplo em maça, uva, jabuticaba, berinjela e mirtilo.

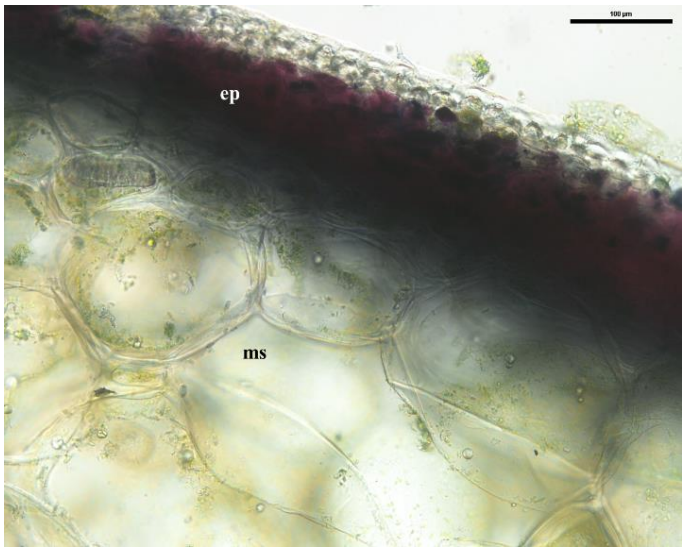

Figura 8. Corte histológico do fruto maduro *Aft/atv/hp2*. Epicarpo (ep); mesocarpo (ms).

CONCLUSÕES

A luz parece ser o principal regulador da ativação de genes responsáveis pela biossíntese de antocianina em frutos *Aft/atv/hp2.*

Plantas *Aft/atv/hp2* possuem desenvolvimento retardado em comparação a plantas selvagens (Micro-Tom), entretanto, frutos maduros de ambos genótipos não diferem em tamanho, mas sim em pigmentação.

Frutos *Aft/atv/hp2* começam a acumular antocianina durante a senescência floral. Além disso, o acúmulo de antocianina acontece especificamente em células do epicarpo.

Plantas *Aft/atv/hp2* parecem ser mais tolerantes ao ataque de trips (Thysanoptera).

Apesar dos relatos feitos nesse trabalho, estudos mais detalhados são necessários para comprovar algumas observações relatadas neste trabalho como o retardamento no desenvolvimento de plantas mutantes, a influência da luz na ativação da biossíntese de antocianina em frutos mutantes tal como a tolerância desse genótipo ao ataque de trips.

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ARTIGO 3: Análise funcional dos genes reguladores da biossíntese de antocianinas em frutos de tomateiro: construção de vetores para superexpressão concomitante de *SLTT8* e *SLMYB114* e knockout de *SLMYB114*

A ser submetido para PLOS ONE JOURNAL

Análise funcional dos genes reguladores da biossíntese de antocianinas em frutos de tomateiro: construção de vetores para superexpressão concomitante de *SLTT8* e *SLMYB114* e knockout de *SLMYB114*

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RESUMO

Técnicas de transformação de plantas tem aberto novos caminhos a serem explorados não só no melhoramento vegetal, mas também em estudos e pesquisas de desenvolvimento e crescimento vegetal, metabolismo, bioquímica, regulação e expressão de genes específicos. Ademais, plantas transgênicas podem ser utilizadas para extração de produtos medicinais, vacinas transgênicas e produção de anticorpos e metabólitos de interesse específico. O acúmulo de compostos secundários benéficos à saúde também tem sido alvo de pesquisas, como por exemplo compostos fenólicos em grãos, frutas e hortaliças. Dentre os compostos secundários que são alvos de pesquisas, a antocianina tem se destacado nos últimos anos, sendo seus benefícios à saúde já amplamente comprovados. Uma vez que a antocianina atribui vários benefícios à saúde e o tomate é um dos legumes mas consumidos no mundo, é de grande valia o acúmulo de antocianina nesse fruto. Dessa forma, pesquisas têm visado aumentar o acúmulo de antocianinas em tomates, tanto por meio de transgenia, quanto por meio de cruzamentos convencionais. Atualmente ainda não está totalmente elucidado quais são os genes responsáveis pela regulação da biossíntese de antocianina em frutos de tomateiro, sendo que este conhecimento é de primordial importância para o avanço das pesquisas nessa área. Visto isso, recentemente um trabalho foi realizado afim de definir os genes responsáveis pela regulação da biossíntese de antocianina em tomates. Os autores utilizaram como objeto de estudo frutos de tomate roxo (*Aft/atv/hp2*), que acumulam antocianina no epicarpo. Por meio de ensaio de qRT-PCR, os autores encontraram dois genes provavelmente responsáveis pelo acúmulo de antocianina em tomates, sendo eles: *SlTT8/ Solyc09g065100* (bHlH) e *SlMYB114/ Solyc10g086290* (R2R3-MYB). Neste sentido, o objetivo com esse trabalho foi construir vetores para futura

transformação de tomateiro. Foram construídos dois vetores: i) visando a superexpresão concomitante de *SlTT8* e *SlMYB114* em tomates vermelhos. ii) visando o knockout de *SlMYB114* por meio da tecnologia CRISPR/Cas9 em tomates roxos.

Palavras-chave: *Solanum lycopersicum*, Superexpressão, CrisprCas9, Tomate roxo.

ABSTRACT

Plant transformation techniques have new paths to be explored not only in plant breeding, but also in studies and research on plant growth and growth, metabolism, biochemistry, regulation and expression of specific genes. In addition, transgenic plants can be used for the extraction of medicinal products, transgenic vaccines and the production of antibodies and metabolites of specific interest. The accumulation of secondary beneficial compounds for health has also been the subject of research, such as the phenomena in grains, fruits and vegetables. Among the secondary compounds that are the target of research, anthocyanin has been outstanding in recent years, and its health benefits have already been widely proven. Since anthocyanin attributes several health benefits and tomatoes are one of the most consumed vegetables in the world, it is of great value or accumulation of anthocyanin in this fruit. Thus, research has aimed to increase the size of anthocyanins in tomatoes, both by means of transgenes, and by means of conventional crosses. It is still not fully elucidated what genes are responsible for the regulation of anthocyanin biosynthesis in tomato fruits, and this is a data of paramount importance for the advancement of research in the area. Seen now, a work has recently been carried out to define the genes responsible for the regulation of anthocyanin biosynthesis in tomatoes. The authors used purple tomato fruits *(Aft/atv/hp2),* which accumulate anthocyanin in the epicarp. By means of qRT-PCR assay, the authors found two genes transferred by accumulation of anthocyanin in tomatoes, being: SlTT8/Solyc09g065100 (bHlH) and SlMYB114/ Solyc10g086290 (R2R3-MYB). The objective with this work was to construct vectors for future tomato transformation. Two vectors were constructed: i) aiming at the concomitant overexpression of SlTT8 and

SlMYB114 in red tomatoes. ii) aiming at the SlMYB114 knockout using CRISPR/Cas9 technology in purple tomatoes.

Key words: *Solanum lycopersicum*, Overexpression, CrisprCas9, Purple tomato.

INTRODUÇÃO

Atualmente, os desafios críticos no uso de recursos hídricos e do solo, bem como as alterações climáticas, estão resultando na escassez dos produtos agrícolas disponíveis. Ademais, é estimado que a população mundial deve chegar a quase 10 bilhões de pessoas em 2050 [1]. Neste sentido, cientistas e pesquisadores vêm pesquisando formas de aumentar tanto a quantidade quanto a qualidade dos alimentos, afim de contribuir para sustentar a população futura.

Por muitos anos o melhoramento convencional de plantas, por meio de cruzamentos e seleção de fenótipos foi o único modo de se obter variedades de plantas mais produtivas com valor comercial e nutritivo agregado. Entretanto, atualmente os alimentos geneticamente modificados (GM) também podem ser uma opção para se realizar o melhoramento de plantas com características específicas desejáveis [2] e para estudos e pesquisas sobre funcionalidade de genes específicos [3]. Estudos com plantas geneticamente modificadas têm relatado a introdução de características, como por exemplo: i) produção de anticorpos, como no caso do anticorpo para HIV 2G12, já confirmada em milho, cevada e arroz [4-6]; ii) melhor eficiência fotossintética e consequente aumento de produção em tabaco [7]; iii) tolerância à deficiência hídrica em alfafa [8], ameixa [9] e soja [10], e tolerância a solos salinos em tomate [11].

Já se tem estudos relatando também o melhoramento nutricional de alimentos, como por exemplo: maior acúmulo de vitaminas em culturas como batata [12], arroz [13], tomate [14], milho [15] e trigo [16]. Também já foram relatados maior acúmulo de ácido fólico em arroz e tomate [17], selênio em mostarda [18], cálcio em batata e alface [19,20] e zinco em arroz [21,22]. O acúmulo de compostos secundários benéficos à saúde também tem sido alvo de

pesquisas, como por exemplo compostos fenólicos em morango e framboesa [23], *Codonopsis lanceolata* [24], sálvia [25], arroz [26], batata [27] e tomate [28-30]. Dentre os compostos fenólicos, a antocianina tem se destacado nos últimos anos, sendo seus benefícios à saúde já amplamente comprovados devido suas atividades biológicas, que incluem propriedades antioxidantes e inflamatórias [31], inibição da oxidação do LDL [32], diminuição dos riscos de doenças cardiovasculares [33], câncer [29,34] e promovendo a acuidade visual, além de ajudar na prevenção da obesidade e diabetes [35].

Uma vez que o acúmulo de antocianinas em alimentos confere diversos benefícios à saúde, o enriquecimento de alimentos com esse pigmento é de grande vantagem para os consumidores. Neste sentido, o tomate se torna um excelente alvo para tal enriquecimento, já que esse fruto é uma das hortaliças mais consumidas no mundo [36]. Pensando nisso, pesquisadores vêm tentando aumentar a concentração de antocianina em tomates, tanto por meio de transgenia [29] como por melhoramento convencional [37].

Algumas pesquisas foram realizadas afim de elucidar os genes responsáveis pela regulação de biossíntese de antocianina em tomates. Porém, os resultados das pesquisas realizadas ainda permanecem incongruentes [38,39]. Recentemente foi verificado por meio de qRT-PCR que dois fatores de transcrição possivelmente os responsáveis pela regulação da biossíntese de antocianinas em frutos de tomate roxo (*Aft/atv/hp2*), são SlTT8/ Solyc09g065100 (bHlH) e SlMYB114/ Solyc10g086290 (R2R3-MYB) [40].

Neste sentido, técnicas de transformação de plantas podem auxiliar a testar as hipóteses sobre quais os genes responsáveis pela regulação da biossíntese de antocianina em tomate.

JUSTIFICATIVA E CONTEXTO DA PESQUISA

As antocianinas são uma classe de pigmentos fenólicos oriundos do metabolismo secundário das plantas cujo consumo proporciona vários benefícios à saúde humana. Sabendo disso, o acúmulo desse pigmento nos alimentos constantemente consumidos pela população, como o tomate, é de grande benefício para a saúde geral da população.

Infelizmente, frutos da espécie *Solanum lycopersicum* normalmente não produzem altos níveis de antocianinas. No entanto, algumas espécies selvagens de tomateiro, como *Solanum lycopersicoides*, *S. cheesmaniae* e *S. chilense* apresentam frutos consideravelmente pigmentados. O locus *Anthocyanin fruit* (*Aft*), introgredido a partir de *S. chilense,* confere frutos parcialmente roxos devido ao acúmulo de antocianinas no epicarpo e outros tecidos do pericarpo, cuja síntese é fortemente modulada pela luz [41]. Dois fatores de transcrição R2R3 MYB localizados no cromossomo 10 são tidos como principais candidatos responsáveis por esse fenótipo: *ANT1* (*SlMYB113/* Solyc10g086260) e *AN2* (*SlMYB75*/ Solyc10g086250) [42,43]. Ademais, Sapir e colaboradores (2008) demonstram que o alelo *high pigment-1* (*hp-1*) interage de maneira sinérgica com *Aft* para aumentar os níveis de flavonóides e antocianinas nos tecidos do pericarpo [44]. Além de *hp-1,* o alelo recessivo *atroviolacea* (*atv*), derivado de *S. cheesmaniae*, atua de forma aditiva ao locus *Aft* [41] aumentando a sensibilidade à incidência de luz vermelha e estimulando fortemente a produção de antocianinas, particularmente nos tecidos vegetativos [45]. De fato, o alelo *atv* exerce influência principalmente sobre a expressão dos genes envolvidos com a síntese geral de flavonóides, como *PHENYLANINE AMONIUM LYASE* (*PAL*), *4-COUMARATO-COA LIGASE* (*4CL*), *CHALCONA ISOMERASE* (*CHI*), e *FLAVANONA 3-HIDROXILASE* (*F3H*), enquanto a transcrição dos genes mais especificamente ligados à síntese de antocianina é modulada por *Aft* [38]. Em mutante duplo *Aft*/*Aft*

atv/*atv*, foi-se observado que todos os genes componentes da via das antocianinas, tanto genes estruturais iniciais (*Early Biosynthetic Genes* - *EBGs*), e principalmente os genes estruturais tardios (*Late Biosynthetic Genes* - *LBGs*), apresentam taxas de transcrição mais elevadas do que em seus correspondentes nas linhagens parentais com mutações simples [38]. No entanto, embora a caracterização genética das plantas mutantes simples *Aft* e *atv*, como do duplo mutante *Aft*/*Aft atv*/*atv* tenha demonstrado que as estratégias de melhoramento clássico são capazes de aumentar o acúmulo de antocianina em tomate, os níveis de pigmentação alcançados foram subótimos e o tamanho dos frutos foi negativamente relacionado com o acúmulo de antocianina [41]. Butelli e colaboradores [29] obtiveram tomates cv. Micro-Tom com níveis substancialmente elevados de antocianinas por meio da transformação genética de dois fatores de transcrição derivados de *Antirrhinum majus*, *DELILA* (*Del*) e *ROSEA1* (*Ros1*). A inserção desses genes heterólogos induziu a produção de antocianina em níveis altos em todos os tecidos dos frutos, inclusive no mesocarpo, demonstrando que a via biossintética da antocianina pode ser ativada integralmente nos frutos de tomate, bem como os níveis de substrato não são limitantes para o acúmulo de antocianinas nos tecidos dos frutos [29]. *Del* é um fator transcrição bHLH indispensável para a ativação dos genes tardios de biossíntese de antocianina incluindo *F3H*, *DFR*, *ANS* e *UGFT* em *Antirrhinum majus* [46]. *Ros1* é um regulador de transcrição do tipo R2R3 MYB que determina os padrões e intensidade de pigmentação das pétalas, modulando a expressão de diferentes genes estruturais da via das antocianinas nessa espécie. A proteína DEL se liga independentemente ao motivo G-box da região promotora de seus genes alvos ou em conjunto com ROS1 e fatores WD-40, formando o complexo MBW [47].

Em tomateiro, esses dois fatores de transcrição aumentaram os níveis de transcritos de *PAL* e de quase todos os genes que codificam as enzimas

catalizadoras da biossíntese de antocianinas, inclusive *CHI* e *F3H*, enzimas chaves no direcionamento da síntese de antocianinas a partir da chalcona [29]. Diferente de Butelli, Sestari *et al.* produziram recentemente por meio de cruzamento convencional um mutante de tomateiro triplo (*Aft/atv/hp2*) cujos frutos roxos apresentam grande acúmulo de antocianinas sem se observar qualquer perda de produtividade [37]. Ademais, os frutos também se mostraram mais ricos em vitamina C e licopeno, enquanto as plantas não tiveram qualquer perda em produtividade [37].

O alelo dominante *Aft* foi introgredido em *Lycopersicon esculentum* (UC82B) a partir de tomateiros *Lycopersicon esculentum (LA1996),* [48]. Alguns trabalhos reportam que a identidade gênica do locus *AFT* se deva a um de dois fatores de transcrição do tipo MYB ligados geneticamente, ANT1 (SlMYB113/ *Solyc10g086260*) [42] ou AN2 (SlMYB75/ *Solyc10g086250*) [43], ambos localizados no cromossomo 10. Diante disso, Sapir e colaboradores [44] sugeriram que se o SlMYB113 é mesmo o responsável pelo acúmulo de antocianina em plantas *Aft*, diferenças na sequência codante, ou seja, na atividade da proteína, e não alterações transcricionais, estariam por trás desse fenótipo dominante. O material vegetal utilizado neste estudo foi o resultado de cruzamentos entre as cultivares Moneymaker e Ailsa Craig *hp1/hp1* (linhagem materna) e o acesso LA1996 - *S. chilense* (doador de pólen). As plantas F_1 resultantes desse cruzamento foram autopolinizadas e as linhagens contendo os alelos Aft^C (*S. chilense*) e *hp1* em homozigose foram selecionadas para os experimentos [44]. Analisando o perfil transcricional de SlMYB113, Sapir e colaboradores [44] não demonstraram aumento nos níveis de transcrição desse gene no genótipo *Aft*.

Por outro lado, avaliando os níveis de mRNA de *SlMYB113* e *SlMYB75* com microarranjos Affymetrix ao longo do amadurecimento dos frutos mutantes duplos *Aft/Aft atv/atv*, Povero e colaboradores [38] relatam que os dois genes são

expressos na epiderme de tomates pigmentados por antocianina. Segundo os autores, ambos os genes apresentam pico de expressão durante a fase verde dos frutos e declínio nas fases 'turning' e maduro, sendo que nenhuma expressão de *ANT1* (*SlMYB113*) foi detectada nessa última fase. Tal padrão é curioso, uma vez que o conteúdo de antocianinas aumenta ao longo do amadurecimento nos frutos desses mutantes. Os duplos mutantes avaliados nesse trabalho foram obtidos por meio do cruzamento dos mutantes simples *Aft/Aft* (LA1996) e *atv/atv* (LA0797). Na falta de linhas isogênicas para ambas as mutações em variedades de *S. lycopersicum*, a cultivar Ailsa Craig (LA2838A) foi escolhida como uma linhagem controle para as análises, o que não é ideal. Também, à luz da sequência genômica do tomateiro, uma análise mais detalhada dos primers usados para a análise transcricional deste trabalho revela que eles não diferem adequadamente os dois alelos ligados potencialmente envolvidos no fenótipo do locus *Aft* (**Figura 3**). Ademais, a qualidade geral dos dados de expressão neste trabalho é dúbia, devido a vários fatores possíveis, como qualidade do RNA utilizado ou mesmo normalização dos dados de expressão.

Em um estudo inicialmente desenhado para estimar a distância de recombinação entre os genes *ANT1* e *AN2*, [39], os autores encontraram um indivíduo *Aft* recombinante com genótipo $ANT1^{C}$ / $ANT1^{C}$ $AN2^{C}$ /$AN2^{L}$, em que $ANT1^{C}$ e $AN2^{C}$ são alelos originados de *S. chilense* e $ANT1^{L}$ e $AN2^{L}$ são alelos oriundos de *S. lycopersicum*. Com base na geração derivada da autopolinização dessa única planta, os autores afirmaram que o fenótipo *Aft* é controlado por um único gene dominante e que esse seria o gene *ANT1* (*SlMYB113*), e não *AN2* (*SlMYB75*). Assim, somente *ANT1* estaria completamente associado com o fenótipo *Aft* e sozinho, o alelo $ANT1^{C}$ geraria o fenótipo característico de acúmulo de antocianina nos frutos. Nesse trabalho, tomates da cultivar Moneymaker foram independentemente transformados com as construções 35S::$ANT1^{C}$ e 35S::$ANT1^{L}$ [39], resultando em plantas transgênicas superexpressado $ANT1^{C}$ e

ANT1^{L}, respectivamente. Entretanto, este teste não é adequado no caso de a diferença alélica se dever ao padrão de expressão de cada gene, ao invés da funcionalidade da proteína *per se*.

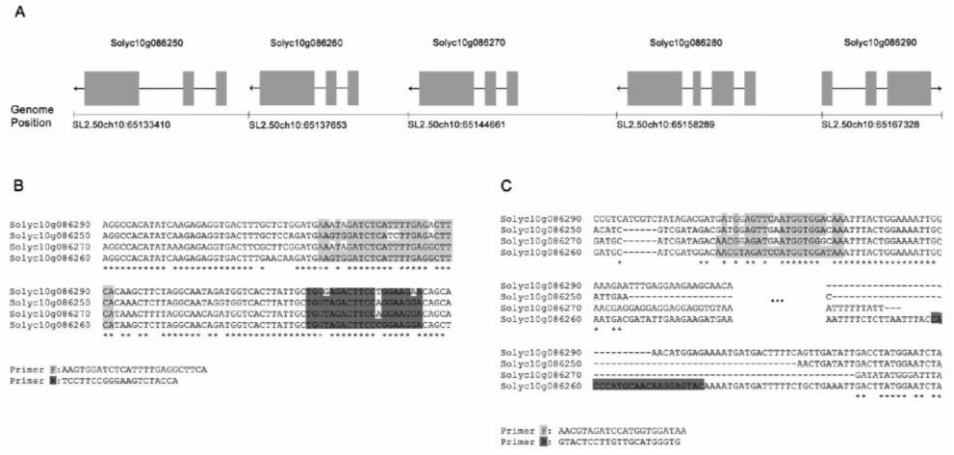


Figura 3. A) Visualização genômica da região de interesse. Os genes *Solyc10g086250* (*SlMYB75*), *Solyc10g086260* (*SlMYB113*), *Solyc10g086270* (*SlMYB28*), *Solyc10g086280* (Proteína transportadora de metais pesados) e *Solyc10g086290* (*SlMYB114*) estão representados. Dentre os cinco genes dessa região de 35 Kb representada, somente *Solyc10g086280* não pertence à família MYB**. B)** Primers desenhados por Povero et al. (2011) para amplificação de *ANT1*/*SlMYB113* (*Solyc10g086260*). **C)** Primers desenhados por Chaves (2015) para amplificação de *ANT1*/*SlMYB113* (*Solyc10g086260*). Somente as regiões de amplificação estão indicadas em B e C. A identidade de nucleotídeos entre os *primers* desenhados e os outros transcritos MYB da região genômica está indicada nas regiões sombreadas.

Devido aos resultados divergentes na literatura com relação à identidade do lócus *Aft*, e para melhor entendimento da regulação transcricional da síntese de antocianina em frutos de tomate, recentemente [40] ensaios de QRT-PCR foram realizados em frutos de tomate roxo oriundos de mutantes triplo introgredidos na cv. Micro-Tom (*Aft*/*atv*/*hp2*) [37] afim de definir quais genes são responsáveis pela regulação da síntese de antocianina em frutos de tomate. [40] utilizou primers

específicos para transcritos de quatro genes: *ANT1* (*SlMYB113/ Solyc10g086260)*, *AN2* (*SlMYB75/ Solyc10g086250*), *SlMYB28* (*Solyc10g086270*) e *SlMYB114* (*Solyc10g086290*), devido à proximidade filogenética desses com outros fatores de transcrição já relatados na literatura como reguladores genuínos da biossíntese de antocianina em outras espécies. Os padrões de expressão dos quatro genes foram verificados em folhas, epicarpo e mesocarpo de isolinhas de tomateiros roxo (cv. Micro-Tom *Aft/atv/hp2*) e vermelho (cv. Micro-Tom).

Por meio de análise de expressão, [40] demonstrou que os genes *SlMYB75* e *SlMYB114* se expressam em todos os tecidos e fases de maturação das plantas mutantes, bem como em algumas condições nas plantas selvagens. O padrão de expressão de *SlMYB114* foi condizente com a distribuição de antocianina nos genótipos analisados. Ademais, *SlMYB114* apresentou alta expressão no epicarpo dos frutos, tecido altamente pigmentado no triplo mutante, quando comparados ao mesocarpo e folhas, tecidos com baixa ou nenhuma pigmentação ciânica. Em plantas mutantes na fase madura, a expressão de *SlMYB114* no epicarpo foi mais de 2.500 vezes maior do que a expressão encontrada nas folhas. Em MT, a maior expressão do gene aconteceu na fase verde, onde a expressão foi apenas 20 vezes maior no pericarpo em relação à encontrada em folhas (**Figura 4**).

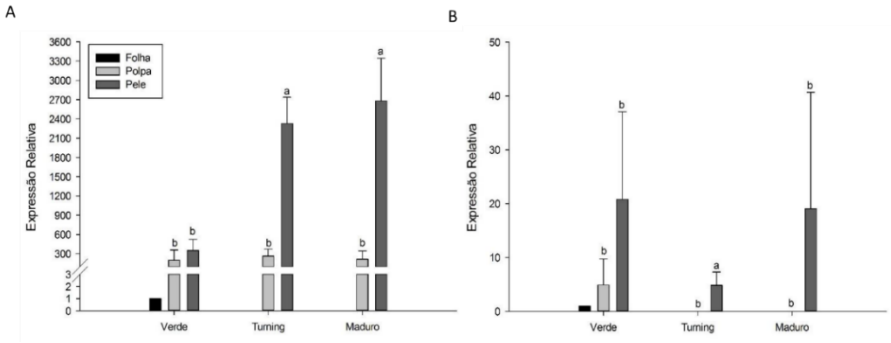


Figura 4. Perfil de expressão relativa de *SlMYB114* (*Solyc10g086290*) em folhas jovens, mesocarpo e epicarpo de tomateiros mutantes MT-*Aft/atv/hp2* **(A)** e em tomateiros selvagens (MT) **(B)**. *Fonte:* [40].

SlMYB75 (*AN2*) foi mais expresso em plantas mutantes do que em plantas selvagens, porém, seus níveis de expressão foram muitos menores do que o encontrado para *SlMYB114*. A maior atividade do *SlMYB75* aconteceu na fase madura, com expressão quase 15 vezes maior no mesocarpo do que em folhas. No epicarpo, o aumento da expressão foi menor que cinco vezes **(Figura 5).**

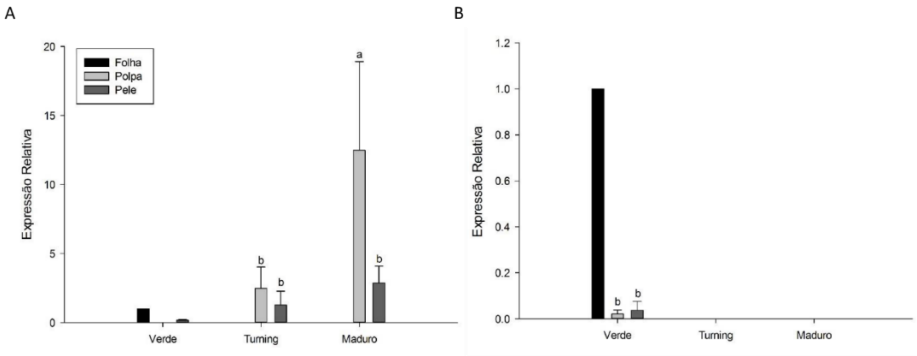


Figura 5. Perfil de expressão relativa de *SlMYB75* (*Solyc10g086250*) em folhas jovens, mesocarpo e epicarpo de tomateiros mutantes MT-*Aft/atv/hp2* **(A)** e em tomateiros selvagens (MT) **(B)**. *Fonte:* [40].

Quanto ao gene *SlMYB28*, nenhuma expressão do gene foi detectada nos experimentos utilizando cDNA sintetizado a partir das folhas, epicarpo ou mesocarpo dos frutos mutantes *Aft/atv/hp2* em nenhum estágio de maturação avaliado (**Figura 6**). Importantly, *SlMYB113*, atualmente tido como o responsável pelo locus *Aft*, não apresentou expressão em nenhum tecido tanto no tomateiro triplo quanto no tipo selvagem.

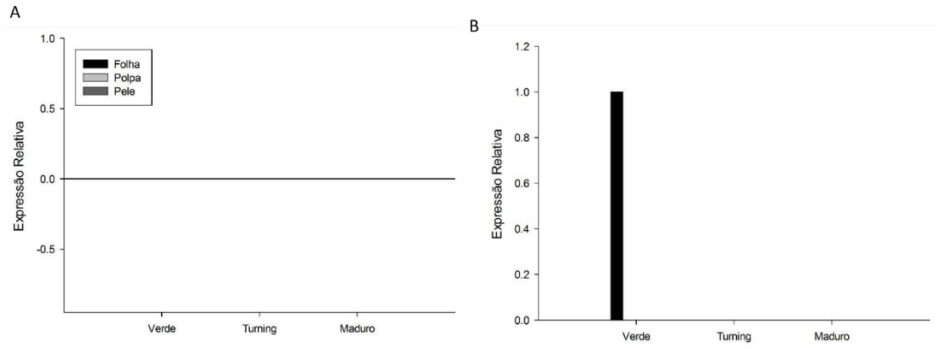


Figura 6. Perfil de expressão relativa de *SlMYB28* (*Solyc10g086270*) em folhas jovens, mesocarpo e epicarpo de tomateiros mutantes MT-*Aft/atv/hp2* **(A)** e em tomateiros selvagens (MT) **(B)**. *Fonte:* [40].

Além da expressão dos quatro genes que transcrevem para fatores de transcrição R2R3 MYB, foi-se avaliado também a expressão de dois genes que transcrevem fatores de transcrição bHLH, *Solyc08g081140* (*SlbHLH090*), *Solyc09g065100* (*SlbHLH150*) bem como o WD40, *SlTTG1-Like* (*Solyc03g09734).* Segundo [40], a expressão do gene *Solyc08g081140* (*SlbHLH090*) foi detectada de forma constante ao longo do amadurecimento dos frutos, com poucas diferenças entre os genótipos avaliados. Em frutos *Aft/atv/hp2*, *Solyc09g065100* (*SlbHLH150*), é altamente expresso no epicarpo durante a fase madura a expressão nesse tecido chega a ser 1.800 vezes maior do que a encontrada em folhas. Por outro lado, nenhum transcrito foi amplificado nos frutos de plantas selvagens no mesmo estágio de maturação (**Figura 7**).

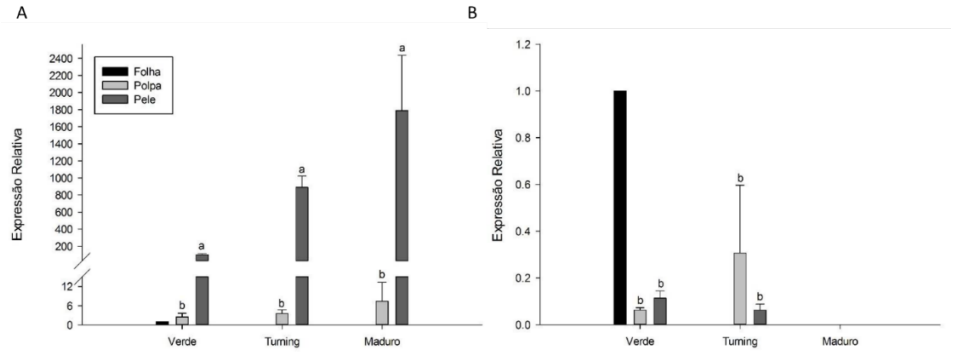


Figura 7. Perfil de expressão relativa de *SlbHLH150* (*Solyc09g065100*) em folhas jovens, mesocarpo e epicarpo de tomateiros mutantes MT-*Aft/atv/hp2* (**A)** e em tomateiros selvagens (MT) **(B)**. *Fonte:[40]*

Somente *Solyc03g097340* foi identificado como o possível homólogo de *TTG1 (WD40)* de *Arabidopsis thaliana* [40]. Foi verificado que nas plantas mutantes a representação do gene é crescente de acordo com o amadurecimento dos frutos. Foram encontradas diferenças significativas entre a expressão do gene no mesocarpo e epicarpo dos frutos coletados na fase '*turning*' e maduro (**Figura 8**). Assim como visto para os genes *SlMYB114* e *SlTT8-like*, o maior nível de transcritos foi encontrado no epicarpo dos tomates roxos em fase final de amadurecimento.

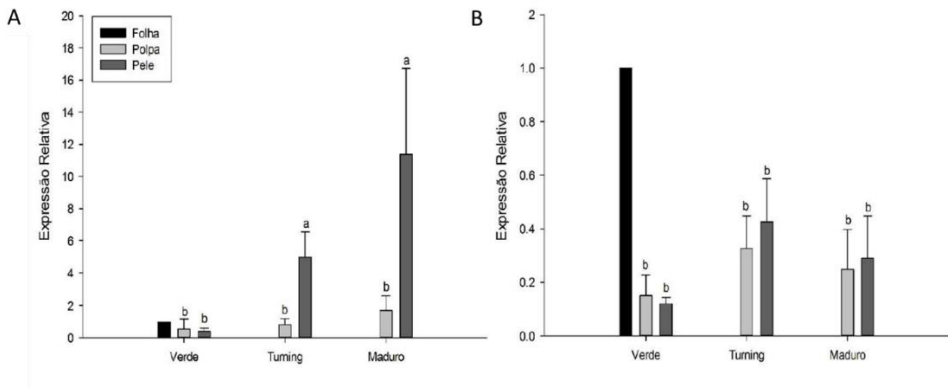


Figura 8. Perfil de expressão relativa de *SlTTG1-Like* (*Solyc03g097340*) em folhas jovens, mesocarpo e epicarpo de tomateiros mutantes MT-*Aft/atv/hp2* (a) e em tipo selvagem (MT) (b). Os níveis dos transcritos são representados como uma razão (expressão relativa) do valor absoluto da expressão do gene alvo pelo valor absoluto da expressão dos genes normalizadores *β-tubulina* e *GAPDH*. Os valores de expressão dos tecidos do fruto foram plotados tendo os valores de expressão encontrados em folhas como referência. Os dados são médias de três amostras biológicas. *Fonte:* [40]

Por meio dos resultados obtidos [40], conclui-se que os prováveis fatores de transcrição (R2R3-MYB e bHLH) que compõem o complexo MBW responsável por regular a síntese de antocianina em tomates são *Solyc10g086290/MYB114* (R2R3 MYB) e *Solyc09g065100/TT8* (bHLH). Tal resultado difere dos relatados anteriormente por Povero *et al.* (2011) e Schreiber *et al.* (2012). Nesse sentido, no intuito de gerar informação útil para o melhoramento de tomate com maior acúmulo de antocianinas, torna-se de grande importância a realização das funções de SlMYB114 e SlTT8 na biossíntese de antocianina em frutos de tomateiro. Um método ideal para responder esta pergunta é através de transformação genética, induzindo ganho ou perda de funções.

OBJETIVOS E HIPÓTESES

Objetivo 1. Construir vetor visando a superexpressão (OX) concomitante de dois genes (*SlTT8* e *SlMYB114*) em tomateiro selvagem (Micro Tom), para transformação genética.

Hipótese 1. Tomateiros transgênicos cv. Micro-Tom resultantes da superexpressão de *SlTT8* + *SlMYB114* produzirão frutos que acumulam antocianinas tanto no epicarpo quanto no endocarpo.

Objetivo 2. Construir um vetor CRISPR Cas9D10A (nickase) visando o knockout (KO) do gene *SlMYB114* no tomateiro mutante triplo (*Aft/atv/hp2*).

Hipótese 2. Tomateiros transgênicos (*Aft/atv/hp2)* em que o gene *SlMYB114* foi silenciado produzirão frutos sem o acúmulo de antocianinas, comprovando seu papel chave na regulação da biossíntese de antocianina em fruto de tomateiro.

REFERENCIAL TEÓRICO

Transformação de plantas

Diferentes espécies do gênero Solanum (seção *Lycopersicon*) vêm sendo utilizadas em programas de melhoramento de tomateiro, visando a introdução de genes que conferem resistência a pragas e doenças, tolerância a estresses abióticos e melhoria da qualidade nutricional e nutracêutica dos frutos [49].

A transformação genética é a transferência de um ou vários genes em um organismo sem necessidade da fecundação ou cruzamento [3], Essa técnica tem aberto novos caminhos a serem explorados não só melhoramento vegetal, mas também permite estudos de desenvolvimento e crescimento vegetal, metabolismo, bioquímica, regulação e expressão de genes específicos. As plantas transgênicas

podem ser utilizadas para extração de produtos medicinais, vacinas transgênicas e produção de anticorpos e metabólitos de interesse específico [50].

As etapas de transformação consistem na identificação do gene de interesse, isolamento do gene de interesse, clonagem, introdução em células ou tecidos vegetais (transformação genética *per se*), seleção *in vitro* de células transformadas, regeneração de plantas transgênicas, confirmação da transgenia por análises moleculares, avaliação em casa de vegetação e testes de campo [51].

A transformação genética de plantas com um gene específico de interesse não implica apenas na transferência desse gene, mas também de alguns elementos que serão necessários para a expressão eficaz do gene na planta. Dessa forma, as sequências que serão inseridas nas células vegetais podem ser chamadas de gene recombinante, DNA recombinante ou construção recombinante. Tal construção necessita conter uma sequência promotora, seguido do gene de interesse, uma sequência terminadora e um gene marcador [50] (**Figura 9**).

Quanto a região promotora, a utilização de promotores tecido-específicos é uma estratégia de fundamental importância para direcionar a expressão de transgenes a determinados órgãos. Como exemplo de promotor específico de tomate, pode-se citar o promotor E8, cujo gene ACO está envolvido na produção de etileno em tomate, afetando consequentemente a maturação dos frutos [28,52,53]. Devido à sua especificidade, o promotor E8 é utilizado em trabalhos de engenharia genética em culturas como melão[54], morango [55], bem como o próprio tomate [28,29]. Para além do promotor, os genes quiméricos em eucariotos devem possuir uma sequência de terminação de transcrição após o STOP CÓDON.

O gene quimérico deve ser complementado com um cassete (promotor-região codante-terminador) responsável pela produção de um fator que permita a

seleção ou a identificação das células transformadas. Esses cassetes são chamados de marcadores e podem ser de dois tipos: genes repórteres e genes de seleção. A utilização desses sistemas é importante durante o processo de seleção das células vegetais transformadas, pois apenas um pequeno número é geneticamente transformada [56].

Figura 9 Ilustração de um DNA recombinante e seus elementos.

Enfim, as técnicas de transferência e superexpressão de genes têm sido muito utilizadas em pesquisas de melhoramento genético de plantas e podem contribuir neste trabalho para a definição dos genes que regulam a biossíntese de antocianina em tecidos do fruto de tomateiro.

Além dessas técnicas, a engenharia genética também tem utilizado a métodos para silenciamento e knockout de genes por meio das técnicas de ZFNs (*Zinc-Finger Nucleases*), TALENs (*Transcription Activator-like Effector Nucleases*) e sistema CRISPR/Cas9 (*Clustered Regularly Interspaced Short Palindromic Repeats).* O uso dessa última técnica pelos pesquisadores vem crescendo e revolucionando a área da engenharia genética [57] por permitir a edição de genes especialmente para knockout de funções gênicas [58].

CRISPR (*Clustered Regularly Interspaced Short Palindromic Repeats*)

A edição de genomas tem sido feita tradicionalmente usando metodologias complexas de ZFN (*zinc-finger nucleases*) e TALENs (*transcription activator like effector nucleases*) que utilizam nucleases quiméricas e customizadas para atuar

em sequências específicas de DNA. Recentemente, uma terceira ferramenta foi introduzida: o sistema CRISPR/Cas9, descrito como o primeiro sistema imune adaptativo e herdável descoberto em procariotos [59] e que já foi eficientemente usada para alterar múltiplos genomas, tanto em procariotos, quanto em eucariotos complexos, como fungos, mamíferos e plantas.

Em 1987, Ishino e seus colaboradores descobriram uma estrutura no genoma de bactérias *E. coli***.** Um *locus* constituído por sequências repetidas de 29 pares de bases "interespaçadas" por sequências variáveis de 32 nucleotídeos conhecidas como "espaços". O *locus* foi denominado CRISPR (*clustered regulary interspaced palindromic repeats*) [60].

A princípio, não foi dada muita importância para o CRISPR, até que o advento do sequenciamento dos genomas de bactérias e vírus mostrou que os "espaços" eram sequências compatíveis com fagos e plasmídeos. Alguns anos depois foram descobertas as primeiras evidências de que o CRISPR estava envolvido em um sistema de proteção altamente conservado em procariotos (45% das bactérias e 90% das Archeae contém o loci CRISPR). Como o CRISPR está colocalizado com o gene *Cas* e funcionam em coordenação, este sistema ficou conhecido como CRISPR/Cas [61].

O sistema CRISPR/Cas é dividido em três tipos principais (tipos I, II e III) baseados na filogenia e presença de proteínas nucleases Cas específicas [86]. Uma particularidade do sistema I é a presença de pelo menos um gene que codifica a nuclease Cas3, que participa ativamente na clivagem da molécula de DNA invasor de modo sequência-específica [62]. O sistema II é um dos mais caracterizados [63], nele são encontrados apenas quatro genes, dentre os quais sempre há ao menos um gene que codifica a Cas9, a qual pode participar tanto no processamento de RNA quanto na eliminação do DNA alvo [62,63]. O sistema III foi dividido em dois subtipos. O sistema III-A é capaz de atuar sobre DNA plasmidial *in vivo*, e o sistema III-B é responsável pela clivagem apenas de RNA

fita simples *in vitro*. Estas observações sugerem a existência de diferentes mecanismos de atuação envolvendo subtipos distintos de CRISPR [62,64].

O sistema CRISPR/Cas tipo II é no momento o mais utilizado na engenharia genética para edição de genomas [63]. Ele é composto por uma endonuclease Cas9 e duas moléculas de RNA curtas: i) RNA trans-codificado designado de tracrRNA e ii) CRISPR RNA trans-ativador designado de tracRNA [65,66]. O crRNA é o responsável pela orientação da endonuclease Cas9 para o DNA alvo [66,67] e é obtido a partir da transcrição de pequenos segmentos de DNA exógeno, interligados entre si dentro do locus genômico de CRISPR [68]. Os crRNAs se emparelham com os tracrRNA específicos da sequência, orientando as Cas9 para certos "protospacers" localizados no DNA alvo, conseguindo assim direcionar o knockout [66,68].

O tracrRNA é um componente essencial do complexo ribonucleico do sistema CRISPR/Cas9, atuando como um ativador necessário para processar o crRNA e também como um componente essencial e indispensável do complexo de DNA. Tem-se mostrado ser igualmente um fator importante na função reguladora da expressão em genes endógenos [69]. O crRNA pode ser usado em qualquer tipo do sistema CRISPR/Cas9, enquanto que o tracrRNA apenas pode ser usado pelo tipo II [69]. O reconhecimento da proteína Cas9 requer uma sequência do crRNA (20 nucleotídeos) [67] capaz de dirigir especificamente a nuclease para o seu destino, e de um motivo adjacente ao protospacer (PAM) localizado a jusante da região do crRNA [66]. O sistema CRISPR/Cas9 é assim direcionado para clivar sequências de DNA próxima ao elemento PAM, após a programação do crRNA para o alvo desejado [68].

A maioria dos estudos têm relatado o uso de um único RNA guia (gRNA), sendo este a junção do crRNA programado e do tracrRNA, como no caso do presente trabalho. No entanto, também foi demonstrado que podem atuar

separadamente, mostrando taxas de edição substancialmente mais baixas do que se utilizadas com um único gRNA [67].

Podemos observar os respectivos locais do PAM, crRNA, e tracrRNA de um sistema CRISPR/Cas9 D10A (nickase), onde crRNA e tracrRNA juntos formam o gRNA **(Figura 9)**.

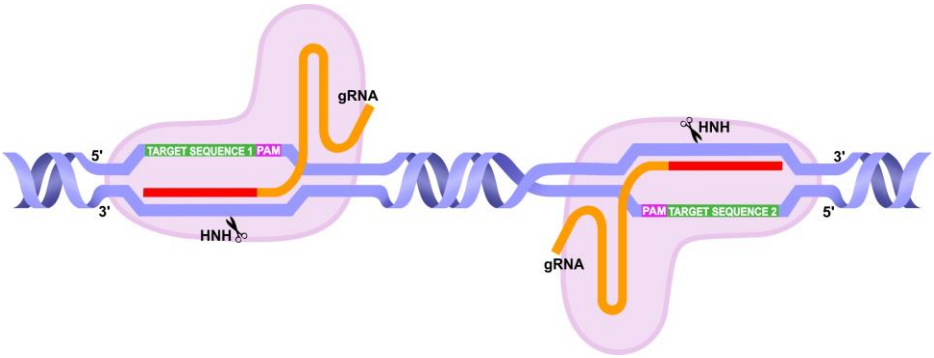


Figura 9. Ilustração de um sistema CRISPR/Cas9 D10A (nickase) contendo: PAM em rosa; crRNA em vermelho e tracrRNA em laranja. A junção do crRNA e tracrRNA forma o gRNA.

Após o reconhecimento e clivagem do DNA alvo, acontece um processo de reparo do DNA clivado que pode ser do tipo NHEJ (*Non-Homologous End Joining*) ou HDR (*Homology-Directed Repair)* [63]. A união de extremidades não-homólogas é o principal mecanismo de reparação de clivagem de fitas duplas na NHEJ. Nesse mecanismo, as extremidades de uma molécula de DNA, são justapostas para recombinar. Desta forma, a sequência original de DNA acaba sendo alterada e frequentemente resulta em integração ilegítima de sequências introduzidas, gerando alterações genômicas imprevisíveis. A NHEJ é referida como "não homóloga" porque as extremidades de ruptura são ligadas diretamente sem a necessidade de um modelo homólogo, em contraste com o reparo dirigido por homologia (HDR), onde as sequências lesionadas ou perdidas nos sítios de clivagem são sintetizadas novamente utilizando uma sequência homóloga ao

longo do genoma [70], mesmo que a DNA polimerase envolvida no processo seja de baixa fidelidade.

Apesar de o sistema de defesa CRISPR ter sido identificado pela primeira vez por Mojica e colaboradores em 2000 [71], apenas em 2013 o sistema CRISPR tipo II observado das *S. thermophilus* e *S. pyogenes* foi desenvolvido. Atualmente, já se tem relatos da utilização da CRISPR-Cas9 para deletar, inserir, ativar ou reprimir genes específicos em bactérias [72], células humanas [73], peixe-zebra [74], ratos [73,75] e, mais recentemente, em plantas [58,76-79]. O que torna esse sistema atraente é o fato de ser uma endonuclease guiada por um gRNA que pode, a princípio, editar qualquer tipo de genoma, desde que presentes todos os elementos requeridos. O sistema CRISPR/Cas9 altamente específico e eficiente [63,80,81] para editar genomas do que as metodologias ZFN e TALENs, pois não necessita de engenharia proteica para marcação dos genes, mas apenas de porções de DNA que permitam codificar um gRNA e uma Cas9, o que dispensa o desenho customizado de novas nucleases para cada novo gene de interesse [63].

Entretanto, a CRISPR/Cas9 também possui algumas desvantagens, como as mutações fora do alvo [58,63]. Numa tentativa de minimizar essa desvantagem, utilizou-se a Cas9nickase (Cas9n) [58,63]. A Cas9n possui uma mutação (de um aspartato para alanina, D10A) em um de seus domínios conservados (RuvC) [63]. Diferentemente da Cas9 que corta a fita dupla de DNA gerando pontas cegas, a Cas9n realiza a clivagem do alvo em dois locais simultaneamente, podendo desse modo, diminuir as mutações fora do alvo [58]. Entretanto, mutações fora do alvo não é de total responsabilidade das Cas9 utilizadas (Cas9 ou Cas9n) mas sim da presença de outros elementos PAM localizados próximos ao PAM alvo [65] [67]. Normalmente a sequência PAM reconhecida pela Cas9 é uma NGG, porém, mas também foi relatada a sequência NAG como alvo [72,82]. Dessa maneira, é de grande importância a escolha do PAM alvo ideal afim de diminuir as mutações fora do alvo. Um exemplo é mostrado na **Figura 10** [58]. Neste trabalho, os

pesquisadores utilizaram a Cas9n, mas mesmo assim observaram grande incidência de mutações fora do alvo. Entretanto, nota-se que as mutações fora do alvo aconteceram em locais em que presentes outros elementos PAM (NGG e NAG) estes estavam aos PAM alvo. Embora os autores não tenham atribuído as mutações fora do alvo à presença de outros elementos PAM, é importante evitá-los sempre que possível. Os resultados ilustrados na **Figura 10** servem como alerta para a escolha dos elementos PAM alvos. Neste sentido, acredita-se que uma boa escolha dos elementos PAM alvos aliada à utilização de uma nickase Ca9n, se consegue uma melhor eficiência no uso da técnica CRISPR diminuindo as mutações fora do alvo.

Enfim, a utilização do sistema CRISPR/Cas9 tem aumentado a cada dia e se mostrado uma ótima ferramenta para o estudo da função de genes por meio do knockout gênico. Nesse sentido, a utilização da CRISPRCas9n no presente trabalho nos ajudará a compreender melhor a função do gene *SlMYB114* na biossíntese de antocianinas em frutos de tomate roxo.

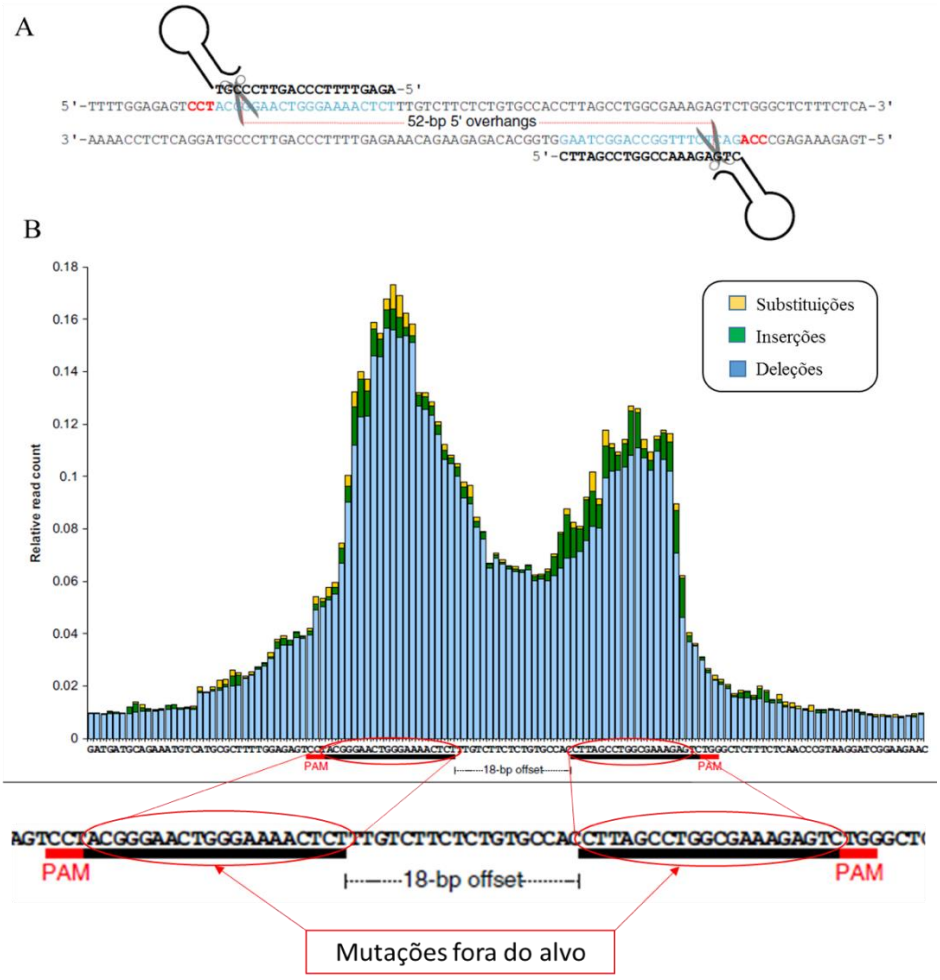


Figura 10. Ilustração das sequências PAM escolhidas por Schiml et al., 2014 e, os previstos locais de cortes (A); Ilustração das mutações fora dos alvos (circulados em vermelho e ampliados), provavelmente devido a presença de outras sequências PAM (NAG e NGG) (B). *Fonte:* [58].

MATERIAL E MÉTODOS

Superexpressão dos genes *SlMYB114* e *SlTT8*

O intuito da superexpressão dos genes *SlMYB114* (*Solyc10g086290*) e *SlTT8* (*Solyc09g065100*) é comprovar sua função na regulação da biossíntese de antocianina em tomates roxos (mutante triplo *Aft/atv/hp2*). O T-DNA do plasmídeo binário (pPLV02_V2+OX) foi composto de quatro módulos (**Figura 11**): **Módulo 1** (vermelho), promotor específico do E8 (pE8) para expressão específica em fruto; **Módulo 2** (azul), cDNA do *SlMYB114* (+ 80 pb de 3' UTR); **Módulo 3** (laranja), promotor E8; e **Módulo 4** (verde), cDNA do *SlTT8* (+ 150 pb de 3' UTR). Após a contrução do DNA recombinante este foi inserido no vetor pPL02_V2 [83], originando o vetor final pPLV02_V2+OX (**Figura 11**). A clonagem e construção do vetor foi realizada em quatro etapas, conforme descrito a seguir.

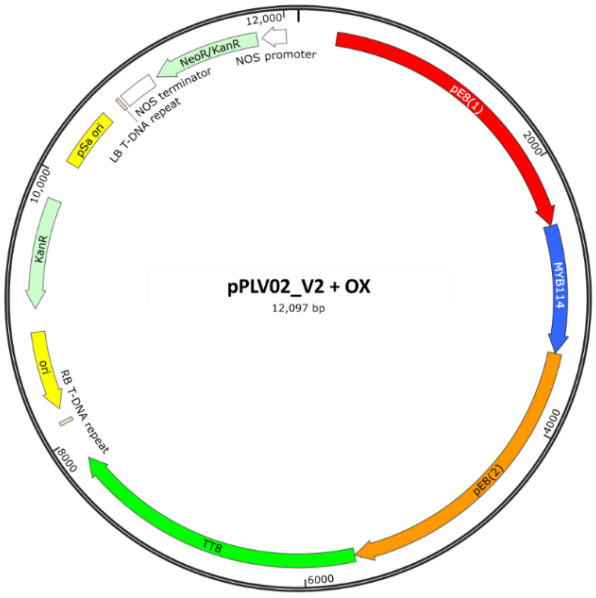


FIGURA 11. Vetor binário final pPLV02_V2+OX. Figura produzida por meio do software SNAPGENE®[84]

a. Obtenção dos módulos

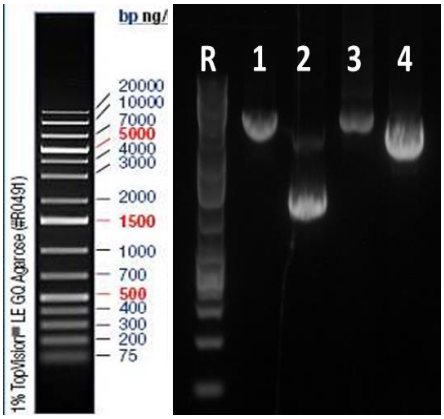
Os módulos 1 e 3 (pE8) foram amplificados por PCR, utilizando DNA genômico (gDNA) de *S. lycopersicum* cv. Micro-Tom. O que diferiu o módulo 1 do módulo 3 foi que este tem 20 nucleotídeos a mais na região 5'-UTR comparado ao módulo 1. Essa diferença foi necessária para o bom funcionamento da técnica (Gibson Assembly®), comentada adiante.

Os módulos 2 e 3 foram amplificados utilizando cDNA obtido do epicarpo de tomates roxos (*Aft/atv/hp2*). A extração de RNA total foi realizada utilizando o reagente TRIzol® -(Life Technologies), o tratamento com Dnase I foi realizado com o TURBO DNase (Life Technologies) e a síntese de cDNA com primers oligo(dT) e o kit SuperScript III reverse transcriptase (Life Technologies), todos esses procedimentos foram realizados segundo o protocolo da empresa. Como descrito anteriormente, ensaios de QRT-PCR realizados por [40] revelaram que tanto SlMYB114 quanto SlTT8 são potenciais reguladores da biossíntese de antocianina em tomates roxos, mostrando uma alta expressão relativa em comparação a tomates vermelhos. Este fato justifica a utilização destes cDNAs para a obtenção dos módulos 2 e 4.

Todos os módulos (1, 2, 3 e 4), foram amplificados por meio de PCR, utilizando a enzima de alta fidelidade KOD Xtreme™ Hot Start DNA Polimerase (Novagen®), segundo o protocolo da empresa. A sequências dos primers utilizados são mostrados na **Tabela 1**. Após a obtenção de todos os módulos, foi realizado uma análise de eletroforese em gel de agarose a 1% com os respectivos módulos (**Figura 12**). Posteriormente, fez-se a eluição dos fragmentos amplificados para cada um dos módulos utilizando o kit de purificação de gel D4002 (Zymoclean™), segundo o protocolo da empresa.

Tabela 1. Primers utilizados para a obtenção dos módulos e seus respectivos tamanhos.

MÓDULOS/ TAMANHO	PRIMERS (Fw) 5' – 3'	PRIMERS (Rv) 5' – 3'
pE8(1)	AAGCTTTCCCTAATGATATT	AATGTCTTGGCAATATTCAT
2203 pb	GTTCATGTAATTAAGTTTTG	GGATCCCTTCTTTTGCACTG
MYB114	ATGAATATTGCCAAGACATT	GTCAAAACATGCTGCTGCAT
878pb	GGGAGTGAGAAAAGGTTCAT	GTCAAAACATGCTGCTGCAT
pE8(2)	ATGCAGCAGCATGTTTTGAC	TTAGGCTGTATAATCTCCAT
2223 pb	AAGCTTTCCCTAATGATATT	GGATCCCTTCTTTTGCACTG
TT8	ATGGAGATTATACAGCCTAA	TCCAACTTGTCCAAGCTTAG
2193 pb	TAGCCTGCAGTTACAAAACA	TCACACATGACTCACTAGAG



.**Figura 12**. Módulos obtidos por meio de PCR R: régua; 1: pE8(1); 2: MYB114; 3: pE8(2); 4: TT8.

b. Junção dos módulos

A junção de todos os módulos amplificados foi realizada pela técnica Gibson Assembly® (New England Biolabs - NEB), segundo o protocolo do fabricante. Essa técnica permite a união de fragmentos por meio da complementariedade dos nucleotídeos nas extremidades de um módulo com os nucleotídeos iniciais

do módulo seguinte (**Figura 13**). O fragmento obtido por meio da junção de todos os módulos (OX: 7.497 pb) foi verificado por meio de eletroforese em gel de agarose a 1% após PCR com o primeiro e último primer (**Figura 14**).

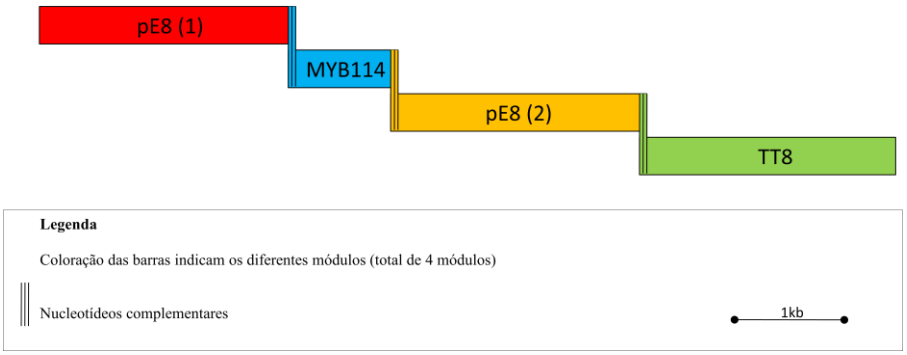


Figura 13. Ilustração dos quatro módulos amplificados e suas respectivas complementariedades.

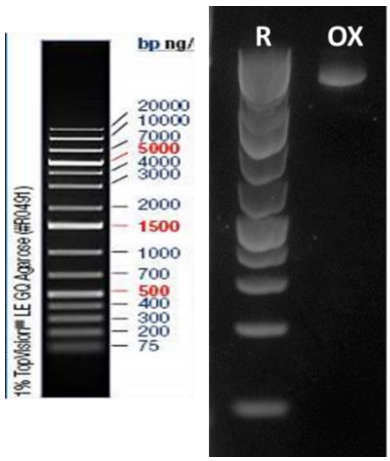


Figura 14. Eletroforese em gel de agarose a 1%. R: Régua; OX: fragmento resultante da junção de todos módulos (pE8(1) + MYB114 + pE8(2) + TT8)

O fragmento OX (**Figura 14**) foi eluído do gel utilizando o kit de purificação D4002 (Zymoclean™), segundo o protocolo da empresa. Em seguida, por meio de PCR (utilizando a enzima KOD Xtreme™ Hot Start DNA Polimerase, Novagen®, segundo o protocolo da empresa), foram adicionados ao fragmento OX sítios de corte da enzima EcoRI (primers na **Tabela 2**), nas extremidades do fragmento, (**Figura 15**). Posteriormente foi realizado uma eletroforese em gel de agarose a 1% e, novamente a eluição do fragmento OX.

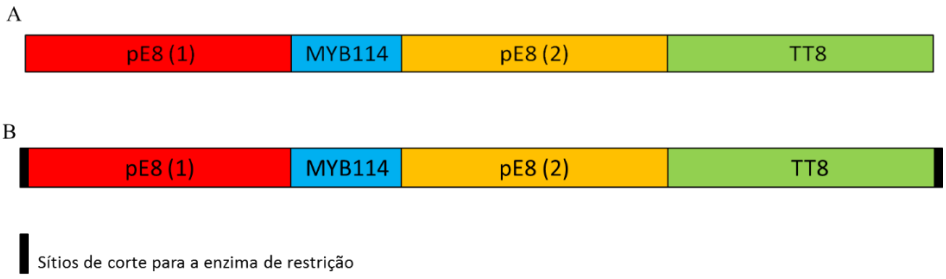


Figura 15. **(A)** Fragmento OX (7.497 pb), após a junção dos módulos sem a inserção dos sítios de corte para a enzima de restrição. **(B)** Fragmento OX após a inserção dos sítios de corte para a enzima de restrição (7.563 pb).

Tabela 2. Primers para inserção de sítios de corte para a enzima de restrição EcoRI

Primer EcoRI (Fw) 5' – 3'	Primer EcoRI (Rv) 5' – 3'
GAGCTC**GAATTC**ATTTTTGACATCCC	CCG**GAATTC**TCCAACTTGTCCAAG CTTAGTCACACATGAC

Legenda: Nucleotídeos em negrito representam o sítio de corte da enzima de restrição EcoRI. Na sequência Fw, 6 pb antecederam o sítio de corte da enzima de restrição, já na sequência Rv, 3 nucleotídeos antecederam o sítio de corte da enzima.

c. Inserção do fragmento OX no vetor pPLV02_V2

O fragmento OX foi inserido no vetor pPLV02_V2 [83] por meio corte com enzima de restrição EcoRI (New England Biolabs) de tanto o fragmento OX quanto o plasmídeo pPLV02_V2, segundo o protocolo da empresa. Após o tratamento com a enzima de restrição, o vetor pPLV02_V2 foi tratado com a Antarctic Phosphatase (New England Biolabs), realizada eletroforese em gel de agarose a 1%, seguido de eluição. A inserção do fragmento OX no plasmídeo pPLV02_02 foi realizada utilizando-se a enzima T4 DNA Ligase (New England Biolabs), dando origem ao vetor pPLV02_V2 + OX.

d. Transformação da bactéria com o vetor pPLV02_V2 + OX

O plasmídeo resultante da inserção do fragmento OX no vetor pPLV02_V2 (OX+pPLV02_V2), foi utilizado para a transformação de bactérias (TOP10®), utilizando o antibiótico de seleção kanamicina (25 mg/L, protocolo em Anexo I). Em seguida, fez-se o miniprep das colônias de bactérias transformadas (protocolo em Anexo II).

Construção do vetor para knockout de SlMYB1114 por meio da técnica CRISPR/Cas9

O vetor final (Knockout_SlMYB114) constitui-se dos seguintes fragmentos: promotor da Ubiquitina 26 de Arabidopsis (pUbi26), nickase Cas9D10A (Cas9D10A); promotor da Ubiquitina 6 de Arabidopsis (pAtU6), sequência alvo 1 (sgRNA1), tracrRNA (sgRNA scaffold); e promotor Ubiquitina 6 de Arabidopisis (pAtU6), sequência alvo 2 (sgRNA2), tracrRNA (sgRNA scaffold), respectivamente (**Figura 16).**

Neste trabalho, escolheu-se a nickase Cas9D10A visando dois cortes simultâneos na fita dupla de DNA. Durante a construção do vetor final (Knockout_SlMYB114), nós utilizamos dois vetores: pEnChimera como vetor de entrada [58] e Cas9D10A como vetor de destino [58]. A construção do vetor Knockout_SlMYB14 foi realizado em 7 etapas.

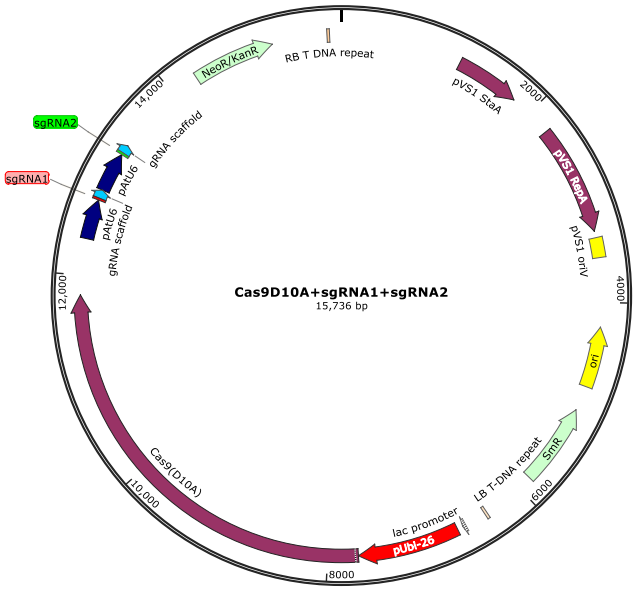


Figura 16: Vetor knokout_SlMYB1114 finalizado com seus respectivos fragmentos. Figura obtida por meio do software SNAPGENE®

a. Obtenção do gene SlMYB114 e escolha dos alvos de edição gênica

A sequência do gene *SlMYB114* foi obtida no site SOL Genomics Network (https://solgenomics.net/). Foram escolhidas duas sequências alvos (sgRNA1 e sgRNA2), ambas sequências localizadas no primeiro éxon do gene *SlMYB114* (**Figura 17**).

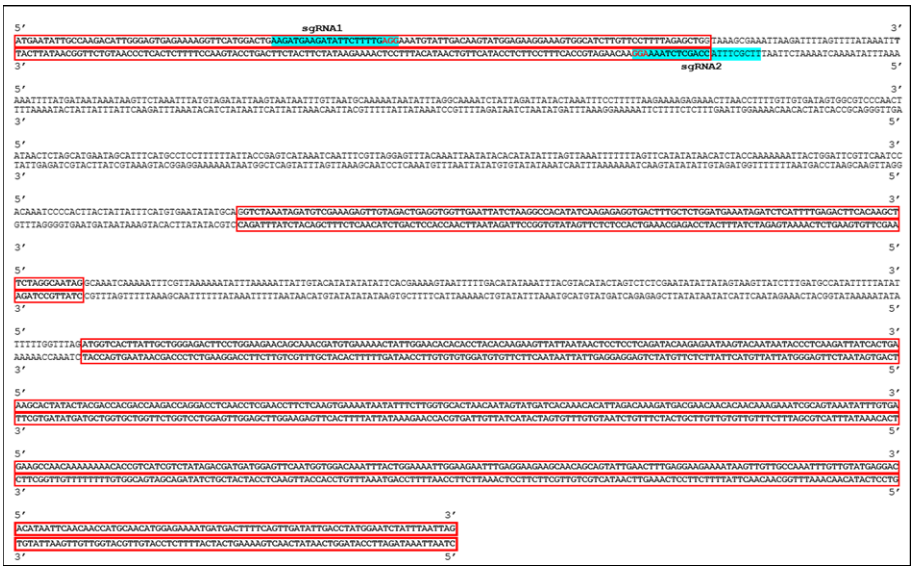


FIGURA 17: Gene SlMYB114. Nucleotídeos circulados em vermelho representam os éxons; nucleotídeos destacados em azul representam os alvos do sgRNA1 e sgRNA2.

b. Obtenção das sequências dos alvos sgRNA1 e sgRNA2 para clonagem

Os fragmentos de DNA para clonagem dos sgRNAs foram sintetizadas artificialmente por meio de primers complementares (24 nucleotídeos), de forma a gerarem um fragmento de DNA em fita dupla com extremidades salientes por anelamento de oligonucleotídeos, segundo Fauser *et al.* [85] para recombinação com DNA processado pela enzima de restrição BbsI. (**Figura 18 A-B**).

c. Digestão do vetor pEn-Chimera e inserção dos sgRNAs

O vetor pEn-Chimera foi digerido com a enzima de restrição BbsI (New England Biolabs), seguido de análise de eletroforese em gel de agarose a 1% (**Figura 18 C-D)**, eluição do gel utilizando com kit de purificação D4002 (Zymoclean^{TM}). Os fragmentos sgRNA1 e sgRNA2 foram clonados em vetores independentes, cada um com seu respectivo sgRNA [pEn-Chimera + sgRNA1 (V1) e pEn-Chimera +

sgRNA2 (V2)] (**Figura 18 E-F**), por meio da técnica de anelamento de oligonucleotídeos, segundo Fauser *et al.* [85].

d. Transformação de bactérias com os vetores

Após as inserções dos sgRNAs foi realizado a transformação das bactérias (TOP 10®) utilizando os respectivos vetores (protocolo no Anexo I). Foi utilizado Carbenicilina (30mg/L) como antibiótico seletivo. Infelizmente, houve crescimento de poucas colônias de bactérias após a transformação, sendo assim, nós produzimos diretamente o miniprep das poucas colônias que cresceram (protocolo em Anexo II) e em seguida a confirmação dos fragmentos inseridos por meio de PCR dos minipreps utilizando a enzima BioReady™ rTaq DNA Polymerase (Bulldog Bio), segundo o protocolo da empresa. O Fragmento de confirmação envolveu o promotor Ubiquiquitina-6 de Arabidópsis (pAtU6), os sgRNAs e a sequência do tracrRNA (sgRNA scaffold), resultando em uma amplificação de 654 pares de base (**Figura 18G**), os primer utilizados podem ser observados na **Tabela 3.** Consideramos que, os fragmentos dos sgRNA são fundamentais para que ocorra o fechamento dos vetores. Assim, uma vez que as bactérias foram transformadas e os fragmentos foram amplificados por meio da PCR (**Figura 18H**), podemos concluir que os fragmentos foram inseridos corretamente nos respectivos vetores.

e. Junção dos dois sgRNA em único vetor

Após a obtenção dos dois vetores iniciais (V1 e V2), o fragmento amplificado do vetor V2 (constituído pelos fragmentos: pAtu6, sgRNA2 e sgRNA scaffold e originando um cassete de 513 pb) pela enzima KOD Xtreme™ DNA polimerase e inserido no vetor V1 (**Figura 19**). Os primers utilizados para a obtenção desse fragmento foram desenhados com o intuído de produzir sítios de corte da enzima XbaI nas extremidades do fragmento. Após a amplificação por PCR, o fragmento foi analisado em gel de agarose (**Figura 20-A**), e eluído com kit de purificação

D4002 (Zymoclean™). O vetor V1 e o fragmento amplificado de V2 foram tratados separadamente com a enzima de restrição XbaI (New England Biolabs) (**Figura 20-B**). V1 foi eluído do gel, as extremidades foram desfosforiladas com Antarctic Phosphatase (New England Biola), purificado com o kit DNA Clean & Concentrator-5 D4014 (Zymoclean™). Realizou-se a junção do fragmento amplificado do vetor V2 com o vetor V1 utilizando a enzima T4 DNA Ligase (New England Biolabs), seguido de transformação de bactérias (TOP10) com o vetor resultante dessa junção (Protocolo I em anexo), utilizando carbenicilina (30mg/L) como antibiótico seletivo. Posteriormente, realizou-se miniprep das colônias que cresceram no meio seletivo (Protocolo II em anexo), seguida de PCR (primers na **Tabela 3**) para confirmar a inserção correta do fragmento V2 havia no vetor V1, seguido de eletroforese em gel de agarose a 1% (**Figura 20-C**).

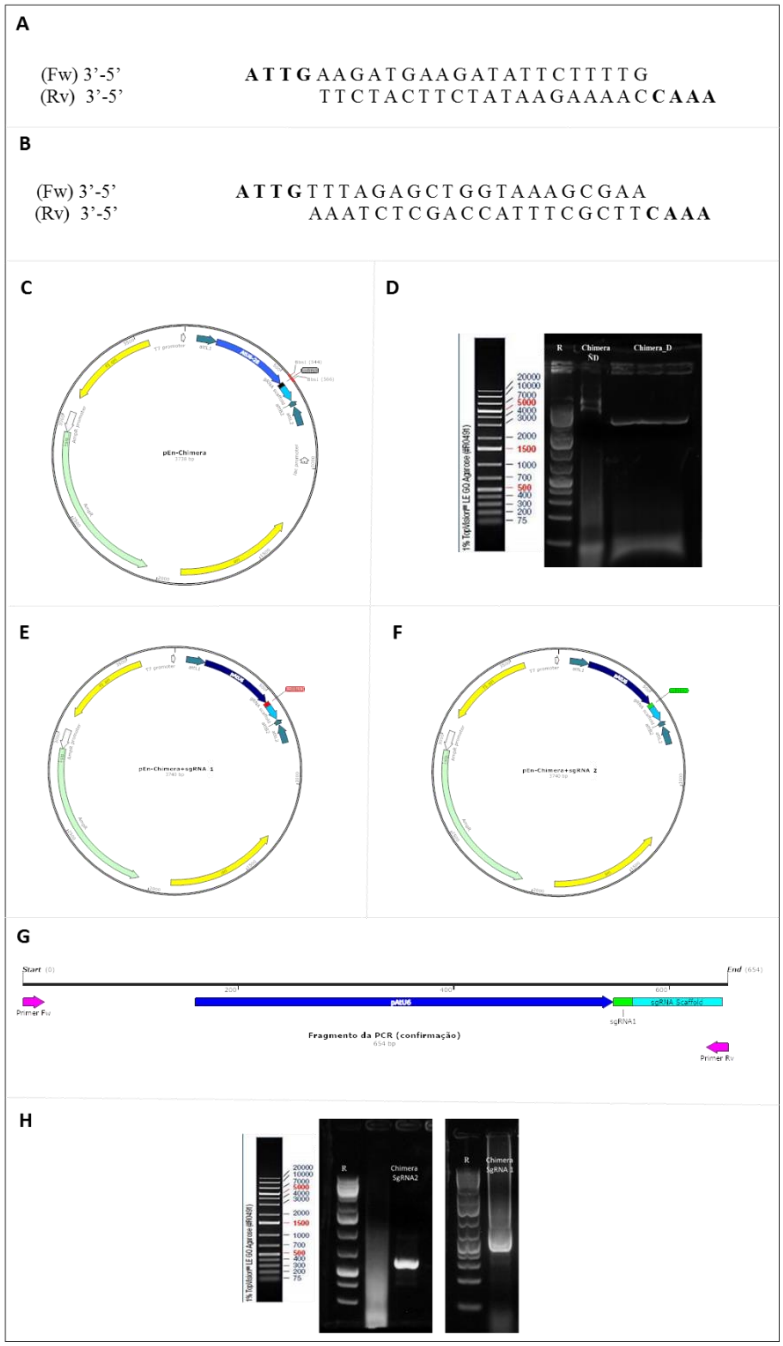


Figura 18: A: Ilustração dos primers referentes ao sgRNA1 após o mix (Fw + Rv). B: Ilustração dos primers referentes ao sgRNA2 após o mix (Fw + Rv). C:

Plasmídeos pEn-Chimera indicando o sítio de corte da enzima de restrição BbsI. D: Gel de eletroforese mostrando Régua (R), pEn-Chimera não digerido (esquerda) e digerido (direita). E: Ilustração da inserção do fragmento sgRNA1 no pEn-Chimera (V1). F: Ilustração da inserção do fragmento sgRNA2 no pEn-Chimera (V2). G: Representação do fragmento replicado pela PCR e analisada em gel. H: Eletroforese dos fragmentos obtidos pela PCR confirmando a inserção dos fragmentos nos respectivos vetores, pEn-Chimera + sgRNA2 (esquerda), pEnChimera + sgRNA1 (direita). Ilustração dos vetores obtidas por meio do software SNAPGENE®

Tabela 3. Primers utilizados para a amplificação de confirmação do sgRNA1 e sgRNA2 (Primers Figura 18-G). Primers utilizados para a amplificação do fragmento do vetor V2 (primers na Figura 19). Primers utilizados para a amplificação de confirmação da inserção de sgRNA1 e sgRNA2 no mesmo vetor.

Primers Figura 18-G	**pAtU6-2-Gib-Fw 5' – 3'**	**Scaffold.XbaI – Rv 5' – 3'**
	GGAATTCGATCAAATAATGA TTTGTACAAAAAAGCAGGCT	GCCGGCGCTTAAGTGATCAC
Primers Figura 19	**XbaI.pAtU6-26-Fw 5' – 3'**	**Scaffold.XbaI-Rv 5' – 3'**
	GTTTCTAGACGACTGATAGTG ACCTGTTCGTTGCAAC	GCCGGCGCTTAAGTGATCAC
Primers Figura 20-C	**XbaI.pAtU6-26-Fw 5' – 3'**	**sgRNA2-Rv 5' – 3'**
	GTTTCTAGACGACTGATAGTG ACCTGTTCGTTGCAAC	AAATCTCGACCATTTCGCTTCA

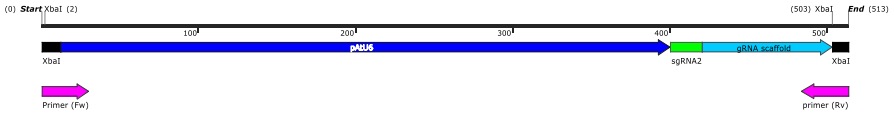


Figura 19: Ilustração do fragmento amplificado de V2 (513 pb), posteriormente inserido no vetor V2. Ilustração do fragmento obtida por meio do software SNAPGENE®

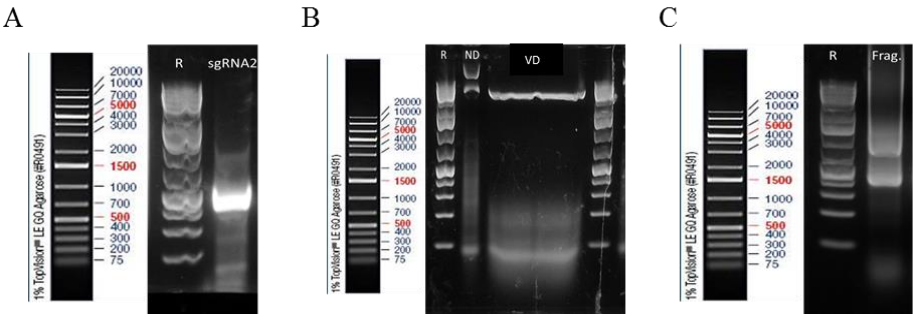


Figura 20. A: Fragmento amplificado de V2 e inserido em V1 (513pb). R: Régua; sgRNA2: fragmento obtido de V2. B: eletroforese de V1. R: Régua; ND: V1 não digerido; VD: V1 digerido. C: Confirmação do fragmento amplificado de V2 inserido em V1, banda maior = 981pb, banda menor 420 pb.

Na **Figura 20-C**, observam-se a presença de duas bandas. Isso se explica pelo fato de o primer foward (fw) utilizado no promotor de ubiquitina 6 (pAtU6), se anelar tanto no fragmento V1 quanto no V2. Entretanto, o primer reverso (rv) utilizado é específico e se anela somente na sequência do sgRNA2 (**Tabela 3**). Assim, a **Figura 20-C** confirma a inserção dos dois sgRNAs (sgRNA1 e sgRNA2) no vetor final (**Figura 21**).

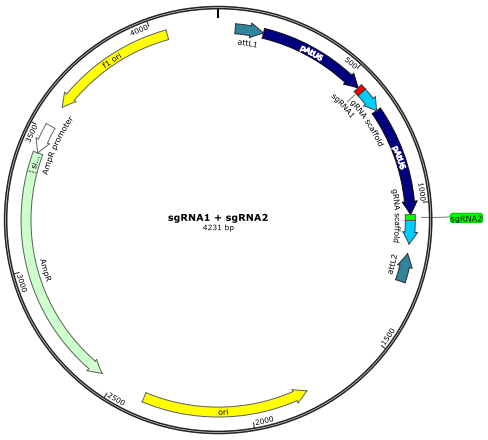


Figura 21: Ilustração do vetor com a junção dos sgRNAs (sgRNA1 +sgRNA2). Figura obtida por meio do software SNAPGENE®

f. Recombinação do vetor sgRNA1+sgRNA2 com o vetor de destino (Cas9 D10A)

Essa recombinação foi realizada utilizando o sistema Gateway® (ThermoFisher Scientific), segundo protocolo do fabricante. O fragmento de DNA de interesse (sgRNA1+sgRNA2), previamente clonado em pEnChimera está flanqueado por dois sítios de recombinação sítio-específica (*attL1* e *attL2*) para ser precisamente transferido para o vetor de destino por reação de recombinação sítio-específica. O vetor de destino utilizado (Cas9D10A) [58], possui sítios correspondentes *attR1* e *attR2* (**Figura 22**). A grande vantagem desse sistema é que, uma vez que o fragmento de DNA foi clonado no vetor de entrada, a transferência para o vetor de destino não requer o uso das tradicionais enzimas de restrição e ligação, mas uma única reação pela LR Clonase II, que catalisa todo processo de recombinação.

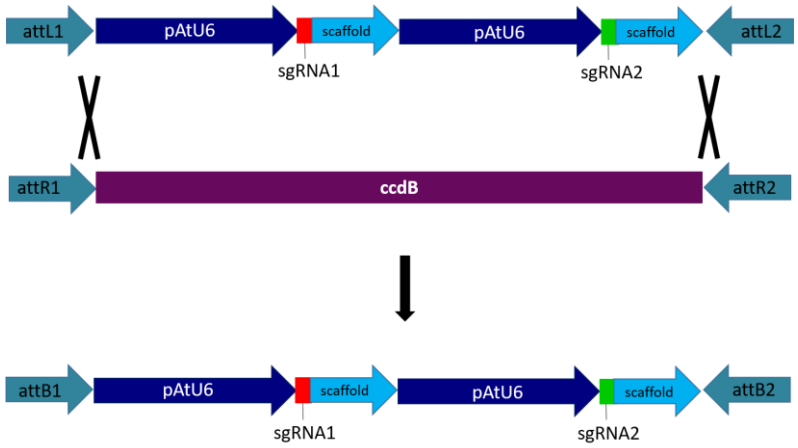


Figura 22. Representação esquemática dos locais *att* e da reação de recombinação Gateway. Em uma reação LR Clonase, os sítios *attL* do vetor de entrada (pEnChimera), recombinam com os sítios *att*R correspondentes do vetor de destino (Cas9 D10A) para se obter *att*B em um novo vetor (Adaptado de Karimi et al., 2007). [86].

g. Obtenção do vetor final (knockout_SlMYB114)

Após a recombinação Gateway® entre os vetores de entrada e destino, deu-se origem ao vetor final, chamado knockout_SlMYB114 (**Figura 23**). Foi realizada a transformação de bactérias (Top10®) (Protocolo I em anexo) utilizando o antibiótico kanamicina (30mg/L) para seleção, seguida de miniprep das colônias resistentes (Protocolo II em anexo) e confirmação da inserção dos fragmentos por PCR com a enzima BioReady™ rTaq DNA Polymerase (Bulldog Bio), segundo protocolo da empresa. Após a PCR, o tamanho dos fragmentos amplificados foram confirmados por eletroforese em gel de agarose (**Figura 24**). Os primers utilizados para a amplificação de confirmação do vetor final foram os mesmos observados na **Tabela 3** e **Figura 20-C**.

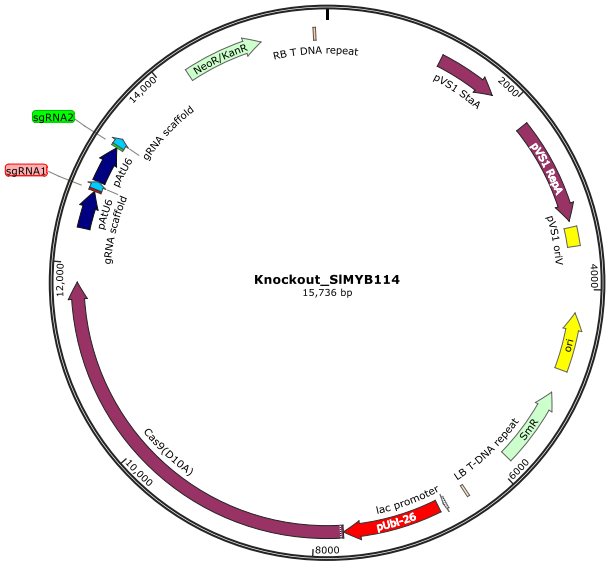


Figura 23. Vetor final após a recombinação por meio do Gateway®. Figura obtida por meio do software SNAPGENE®

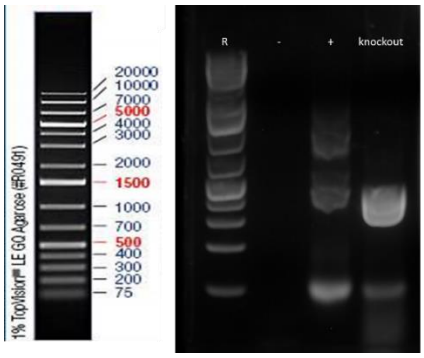


Figura 24. Fragmentos oriundo de PCR. R: Régua; -: controle negativo; +: controle positivo (vetor sgRNA1+sgRNA2); knockout: PCR utilizando o vetor final (knockout_SlMYB114).

6. CONSIDERAÇÕES FINAIS

A montagem do vetor para a superexpressão concomitante de *SlTT8* e *SlMYB114* e do vetor para o knockout de *SlMYB114* por meio do sistema CRISPR/Cas9_D10A (nickase) é um avanço a caminho do nosso objetivo final, que é a transformação de plantas e verificação fenotípica da função dos genes em estudo em relação às suas contribuições na biossíntese de antocianinas em frutos de tomateiro. As próximas etapas para a conclusão da transformação se resumem na introdução dos vetores binários em de *Agrobacterium tumefaciens*, transformação das plantas de tomateiro e fenotipagem.

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ANEXO I

PROTOCOLO PARA TRANSFORMAÇÃO BACTÉRIANA

1. Em microtubo, acrescente a 50μL de células competentes da bactéria *E. coli* TOP10 e 2μL do vetor plasmidial, homogeneizando a solução suavemente.
2. Incube o microtubo em gelo por 30 min.
3. Transfira para banho-maria a 42 °C durante 30 segundos.
4. Transfira rapidamente para o gelo por 2 min.
5. Adicione 250μL de S.O.C. Medium a temperatura ambiente.
6. Incubar o microtubo em movimento horizontalmente a 37°C durante 60 min (200 rpm).
7. Plaquear 10-50μL em placa contendo 20 mL de LB sólido já contendo 10μL do antibiótico seletivo.
8. Incubar a noite toda em 37°C.

ANEXO II

PROTOCOLO PARA PREPARAÇÃO DO MINIPREP

1. Transferir uma colônia bacteriana única para 2 ml de meio LB contendo o antibiótico apropriado num tubo esterilizado. Incubar a cultura durante a noite a 37 °C a 250 rpm.

2. Verter 1,5 ml da cultura num tubo de microcentrífuga. Centrifugar durante 2 minutos a 12.000 g em uma microcentrífuga.

3. Remover o meio, deixando o sedimento bacteriano tão seco quanto possível.

4. Resuspender o sedimento bacteriano (obtido no passo 3 acima) em 200 µl de Solução I gelada por agitação vigorosa.

5. Adicionar 200 µl de Solução II. Feche bem o tubo e misture o conteúdo invertendo o tubo rapidamente. Certifique-se de que toda a superfície do tubo entra em contato com a Solução II. Não vórtice. Guarde o tubo em gelo.

6. Adicionar 200 µl de solução gelada III. Feche o tubo e agite-o suavemente numa posição invertida durante 10s para dispersar a Solução III através do lisado bacteriano viscoso. Armazenar o tubo em gelo por 10 min.

7. Centrifugar a 12.000 g durante 10 minutos numa microcentrífuga. Transferir 1,6µl do sobrenadante para um novo tubo.

8. No novo tubo, adicionar 450µl de isopropanol junto aos 1,6 µl do passo anterior. Misture por vórtex. Após centrifugação a 12.000 g durante 2 minutos numa microcentrífuga, transferir a fase aquosa para um novo tubo.

9. Remover o sobrenadante.

10. Lavar o sedimento de DNA de cadeia dupla com 1 ml de etanol a 70%. Remover o sobrenadante e permitir que o sedimento de ácido nucleico seque no ar durante 10 min.

11. Ressuspender os ácidos nucleicos em 50 µl de TE (pH 8,0). Vortex brevemente. Armazenar o DNA a -20 ° C.

Solução I (50mM TRIS pH 8.0; 10mM EDTA; 0.1 mg/L RNAse A)

Solução II (200mM NaOH; 1% SDS)

Solução III (3M de acetato de potássio gelado, pH 5.5)

ARTIGO 4: Global analysis of the mate gene family of metabolite transporters in tomato

Submitted to: BMC GENOMICS

Global analysis of the mate gene family of metabolite transporters in tomato

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GLOBAL ANALYSIS OF THE MATE GENE FAMILY OF METABOLITE TRANSPORTERS IN TOMATO

ABSTRACT

Species of the Solanaceae family are known for the plethora of secondary metabolites they produce, including alkaloids, phenolics, and terpenoids. In addition to biosynthesis pathways, a comprehensive understanding of secondary metabolism must also take into account the transport and subcellular compartmentalization of substances. Here, we examined the MATE gene family in the tomato (*Solanum lycopersicum*) genome with the objective of furthering our understanding of the transport of secondary metabolites in this species. MATE (Multidrug and Toxic Compound Extrusion or Multi-Antimicrobial Extrusion) membrane transporters encompass an ancient gene family of secondary transporters present in all kingdoms of life, but with a remarkable expansion in plants. Most of the MATE transporters have been characterized to mediate the transport of primary and secondary metabolites using the proton motive force through several membrane systems of the plant cell. We identified 67 genes coding for MATE transporters, of which 33 are expressed constitutively, whereas

34 are selectively induced in specific cell types or environmental conditions. Altogether, our transcriptional data, phylogenetic analyses, and synteny study provide strong evidence of functional homologies between MATE genes of tomato and *Arabidopsis thaliana*. Furthermore, this work sets the stage for genome-wide functional analyses of MATE transporters in tomato as well as in other Solanaceae crop species of economic relevance.

Keywords: antiporter; efflux; genome evolution; plant physiology; transcriptome

Background

The Solanaceae (nightshades) is a botanical family with globally important crops within about 90 genera and ~3,000-4,000 species, such as tomato, potato, eggplant, tobacco, petunia, chilies, and peppers. This family also contains crop species of more local relevance, such as tomatillo, goji, and gooseberry, not to mention countless medicinal, ornamental, toxic, and weed species [1]. Species within this family are known for their prolificacy in producing secondary metabolites, especially alkaloids and also phenolics and terpenoids [2-5]. Given the economic importance as a crop as well as its phenotype and genetic qualities, the tomato (*Solanum lycopersicum*) has been chosen as the biological model species not only for this family, but also for the whole Asterid clade, which comprises of numerous agricultural species, including crops from varied families that produce relevant secondary metabolites, such as stimulant alkaloids in coffee, tea, and yerba mate. This clade also contains important Asteraceae crops (lettuce, sunflower, artichoke, stevia, echinacea, and daisies), and common Lamiaceae herbs (basil, lavender, marjoram, mint, oregano, rosemary, sage, thyme), to name just a few.

Moreover, the choice of the tomato over the well-established rosid model, *Arabidopsis thaliana*, is justified not only by the closer relationship of tomato to other Asteridae crops, but also because of many features of agricultural relevance are not attainable in Arabidopsis, such as development of a complex leaf pattern, climacteric fleshy fruit (botanical berry), establishment of symbiotic root interactions (e.g., mycorrhization) [6], as well as an abundant metabolism of secondary compounds, including alkaloids (e.g., tomatine) [7], phenolics (e.g., rutin, naringenin, apigenin, caffeic acid) [8], and terpenoids (including volatile components of the fruit aroma, such as geranial and norisoprenes) [9, 10].

The availability of the tomato genome sequence and other genetic resources (e.g., molecular markers and genetic maps, germplasm collection, and transcriptional profiles) allows for a global as well as focused analyses of gene functions in order to better understand the developmental and metabolic mechanisms in the species, with the ultimate goal of generating breeding toolkits to improve traits of agricultural relevance [11, 12]. For example, the manipulation of alkaloid transport in the Solanaceae may be key to producing solanine-free potatoes, generating

tomato lines with increased levels of the beneficial glycoalkaloid tomatine, or even the domestication of poisonous wild species for food or feed [13].

MATE (Multidrug and Toxic Compound/Multi-Antimicrobial Extrusion) is a universal gene family of membrane transporters present in all kingdoms of life. However, possibly due to the abundance of secondary metabolites characteristic of plant species, the family has vastly expanded in plant genomes. Most MATEs have 400-550 amino acid residues typically encompassing 12 transmembrane domains (TMD). However, it lacks an absolute conservation of amino acid residue in its core domain sequence (IPR002528/PF01554) [14]. Given the prominent roles in cell detoxification, MATE transporters in Arabidopsis are alternatively called DETOXIFICATION (DTX) [15] . Among the many plant MATE membrane transporters characterized to date, surprisingly very little is known in tomato. Most MATE transporters export primary and secondary metabolites out of the cytosol using electrochemical gradient across the membrane [16], thus mediating the efflux or subcellular compartmentalization of metabolites in the cell (www.tcdb.org) [17]. In plants, MATE transporters have been implicated directly or indirectly in mechanisms of detoxification of noxious compounds or heavy metals [15,

18, 19], tolerance to aluminum toxicity [16, 20-22], disease resistance [23, 24], nutrient homeostasis, such as Fe^{3+} uptake [21, 22, 25], and the transport of diverse types of secondary metabolites, such as alkaloids [26], flavonoids [27, 28] and anthocyanidins [29, 30], as well as hormones, such as ABA, salicylic acid, and auxin [31-33].

Previous research has characterized the functions of the MATE transporters in many species, such as bacteria, yeast, animals and plants [34-39]. Unlike mammalian genomes, which carries only a few MATE genes (e.g., 5 in mouse, 11 in human), plant genomes encode a large number of MATE genes: 56 in Arabidopsis [33] , in rice [40] , 70 in *Medicago truncatula* [22] and 117 in soybean [34]. So far, soybean is the species with the highest number of MATE genes, which can be explained by the high rate of gene duplication of its paleopolyploid genome: 82% of the MATE genes are present in duplicate (thus potentially carrying redundant functions), being 21% arranged in tandem and 61% in large-scale segmental duplications [16]. Since MATE transporters potentially carry essential functions in physiological mechanisms in plants, they could be ideal targets of breeding programs for the improving traits of agricultural relevance, such as aluminum tolerance, iron nutrition, and accumulation of

secondary metabolites of interest (e.g., increase of anthocyanin contents or eradication of toxic alkaloids). A complete analysis of the MATE gene family in a plant species is essential to fully comprehend its secondary metabolism. Herein, aiming to guide future molecular analyses, we identified 67 genes coding for MATE transporters in the tomato genome, and produced a genomic inventory of MATE genes to provide a close look into the functional roles MATE transporters may play in the tomato's physiology and cellular metabolism. We also set a stage for further functional characterization of these MATE transporters as well as manipulation of these traits in relation to plant metabolism.

Results and discussion

Phylogenetic analysis of the MATE gene family in tomato

We identified 67 members of the MATE family of membrane transporters in *S. lycopersicum* (**Table 1** and **Supplemental Table S1**) by using the analysis pipeline previously described [41] and the TransportTP tool [http://bioinfo3.noble.org/transporter] [42]. Phylogenetic analyses of membrane transporters are usually not accurate to assign specific substrates. However, phylogeny of the MATE family has been shown to be

quite useful to predict affinities with potential molecule groups, such as organic acids (e.g., citrate), flavonoids (anthocyanin, proanthocyanidin), and alkaloids (nicotine). Therefore, a phylogenetic analysis was performed with the 67 MATE protein sequences identified in tomato along with 56 from Arabidopsis as well as 33 MATE transporters that were functionally characterized in other plant species (**Figure 1**).

Table 1. Members of the MATE family of membrane transporters (TCDB 2.A.66.1) in the *S. lycopersicum* genome.

Locus	Phylogenetic Clade	Protein size	Transmembrane domains (TMHMM)	Best TCDB match (BLAST)	species	Evalue*	Confidence	Manual curation of classification**
Solyc01g109310	1	476 aa	12	2.A.66.1.44 - TT12	cotton	2.7e-118	100	1
Solyc01g109320	1	497 aa	12	2.A.66.1.44 - TT12	cotton	1.0e-127	100	1
Solyc01g094830	1	1245 aa	12	2.A.66.1.44 - TT12	cotton	9.5e-115	100	1
Solyc03g025200	1	475 aa	12	2.A.66.1.44 - TT12	cotton	2.0e-114	100	1
Solyc03g025210	1	494 aa	12	2.A.66.1.44 - TT12	cotton	6.1e-112	100	1
Solyc03g025240	1	399 aa	12	2.A.66.1.44 - TT12	cotton	2.1e-106	100	1
Solyc03g063730	1	413 aa	12	2.A.66.1.44 - TT12	cotton	7.1e-112	100	1
Solyc03g025190	1	506 aa	12	2.A.66.1.44 - TT12	cotton	3.1e-132	100	1
Solyc03g025220	1	505 aa	9	2.A.66.1.44 - TT12	cotton	3.1e-132	90.9	1
Solyc03g025230	1	500 aa	12	2.A.66.1.44 - TT12	cotton	8.7e-124	100	1
Solyc03g025250	1	501 aa	11	2.A.66.1.44 - TT12	cotton	8.3e-113	100	1
Solyc04g074840	1	503 aa	12	2.A.66.1.44 - TT12	cotton	4.3e-125	100	1
Solyc04g074850	1	482 aa	12	2.A.66.1.44 - TT12	cotton	6.8e-124	100	1
Solyc04g074860	1	569 aa	13	2.A.66.1.44 - TT12	cotton	4.4e-103	100	1
Solyc05g013450	1	515 aa	13	2.A.66.1.44 - TT12	cotton	2.0e-144	100	1
Solyc05g013460	1	514 aa	12	2.A.66.1.44 - TT12	cotton	1.1e-140	100	1

Locus	Phylogenetic Clade	Protein size	Transmembrane domains (TMHMM)	Best TCDB match (BLAST)	species	Evalue*	Confidence	Manual curation of classification**
Solyc05g013470	1	504 aa	12	2.A.66.1.44 - TT12	cotton	3.6e-142	100	1
Solyc06g036130	1	504 aa	12	2.A.66.1.44 - TT12	cotton	4.1e-132	100	1
Solyc07g008410	1	480 aa	11	2.A.66.1.44 - TT12	cotton	2.0e-137	100	1
Solyc10g051130	1	478 aa	12	2.A.66.1.44 - TT12	cotton	3.2e-117	100	1
Solyc10g080340	1	507 aa	12	2.A.66.1.44 - TT12	cotton	1.2e-122	100	1
Solyc10g081260	1	485 aa	11	2.A.66.1.44 - TT12	cotton	8.5e-113	100	1
Solyc11g010380	1	493 aa	11	2.A.66.1.44 - TT12	cotton	3.9e-126	100	1
Solyc12g019320	1	502 aa	12	2.A.66.1.44 - TT12	cotton	5.4e-135	100	1
Solyc02g032660	2	504 aa	12	2.A.66.1.44 - TT12	cotton	2.0e-161	100	1
Solyc02g080480	2	499 aa	12	2.A.66.1.44 - TT12	cotton	6.1e-159	100	1
Solyc02g080490	2	504 aa	12	2.A.66.1.44 - TT12	cotton	6.6e-157	100	1
Solyc07g052380	2	503 aa	12	2.A.66.1.44 - TT12	cotton	1.7e-162	100	1
Solyc12g005850	2	512 aa	12	2.A.66.1.44 - TT12	cotton	1.0e-165	100	1
Solyc12g006360	2	503 aa	12	2.A.66.1.44 - TT12	cotton	0.0	100	1
Solyc02g091050	3	474 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	0.0	100	1
Solyc02g091070	3	471 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	0.0	100	1

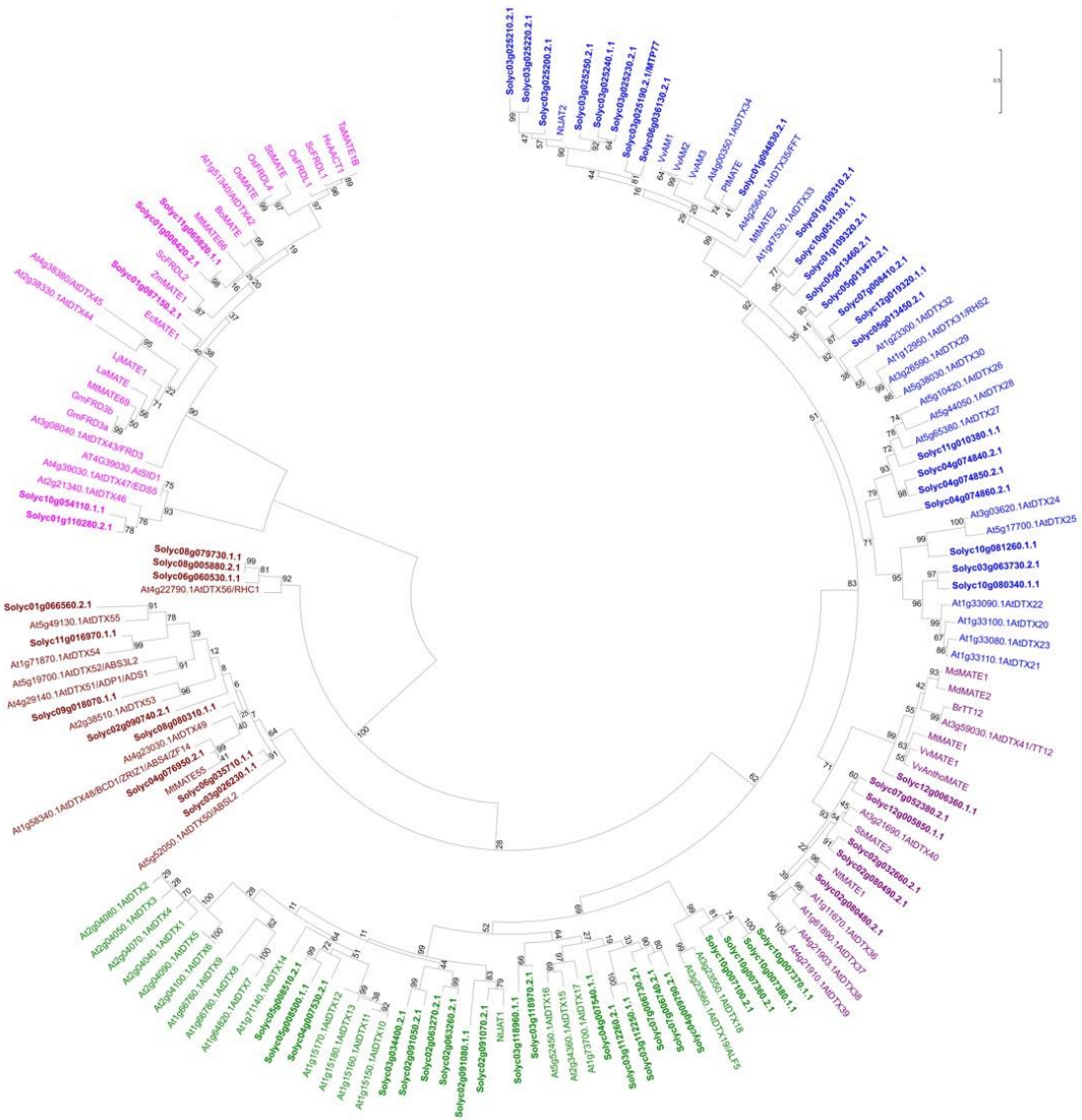
Locus	Phylogenetic Clade	Protein size	Transmembrane domains (TMHMM)	Best TCDB match (BLAST)	species	Evalue*	Confidence	Manual curation of classification**
Solyc02g091080	3	507 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	0.0	100	1
Solyc02g063260	3	492 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	0.0	100	1
Solyc02g063270	3	454 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	0.0	100	1
Solyc03g034400	3	470 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	0.0	100	1
Solyc03g112250	3	489 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	6.8e-136	100	1
Solyc03g112260	3	519 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	7.2e-126	100	1
Solyc03g118960	3	419 aa	8	2.A.66.1.8 - At2g04040	Arabidopsis	2.7e-54	100	2
Solyc03g118970	3	495 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	2.3e-141	100	1
Solyc04g007530	3	481 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	0.0	100	1
Solyc04g007540	3	480 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	6.3e-137	100	1
Solyc04g009790	3	1255 aa	13	2.A.66.1.19 - putative MATE transporter	tobacco	2.2e-127	100	1

Locus	Phylogenetic Clade	Protein size	Transmembrane domains (TMHMM)	Best TCDB match (BLAST)	species	Evalue*	Confidence	Manual curation of classification**
Solyc05g008500	3	456 aa	11	2.A.66.1.19 - putative MATE transporter	tobacco	4.0e-151	100	1
Solyc05g008510	3	478 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	0.0	100	1
Solyc07g006730	3	489 aa	12	2.A.66.1.8 - At2g04040	Arabidopsis	3.3e-135	100	1
Solyc07g006740	3	471 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	3.7e-127	100	1
Solyc10g007100	3	488 aa	11	2.A.66.1.8 - At2g04040	Arabidopsis	0.0	100	1
Solyc10g007360	3	482 aa	12	2.A.66.1.6 - ALF5	Arabidopsis	0.0	100	1
Solyc10g007370	3	547 aa	12	2.A.66.1.6 - ALF5	Arabidopsis	3.9e-175	100	1
Solyc10g007380	3	512 aa	12	2.A.66.1.6 - ALF5	Arabidopsis	2.0e-176	100	1
Solyc01g066560	4	495 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	6.2e-67	100	1
Solyc02g090740	4	505 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	5.7e-77	100	1
Solyc03g026230	4	527 aa	11	2.A.66.1.19 - putative MATE transporter	tobacco	1.2e-84	100	1
Solyc04g076950	4	550 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	5.9e-79	100	1
Solyc06g035710	4	520 aa	13	2.A.66.1.19 - putative MATE transporter	tobacco	1.2e-86	90.9	1

Locus	Phylogenetic Clade	Protein size	Transmembrane domains (TMHMM)	Best TCDB match (BLAST)	species	Evalue*	Confidence	Manual curation of classification**
Solyc06g060530	4	487 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	4.9e-70	100	1
Solyc08g005880	4	534 aa	13	2.A.66.1.44 - TT12	cotton	8.9e-121	100	1
Solyc08g079730	4	480 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	7.6e-78	100	1
Solyc08g080310	4	494 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	2.0e-88	100	1
Solyc09g018070	4	466 aa	12	2.A.66.1.19 - putative MATE transporter	tobacco	1.0e-71	100	1
Solyc11g016970	4	472 aa	11	2.A.66.1.19 - putative MATE transporter	tobacco	5.5e-74	100	1
Solyc01g008420	5	536 aa	12	2.A.66.1.43 - MATE efflux protein	maize	0.0	100	1
Solyc01g087150	5	525 aa	13	2.A.66.1.24 - FRD3	Arabidopsis	0.0	100	1
Solyc01g110280	5	557 aa	11	2.A.66.1.11 – EDS5/SID1	Arabidopsis	0.0	100	1
Solyc10g054110	5	375 aa	6	2.A.66.1.11 – EDS5/SID1	Arabidopsis	1.7e-136	100	2
Solyc11g065820	5	510 aa	12	2.A.66.1.43 - MATE efflux protein	maize	0.0	81.8	1

*Evalue of Blast search on TCDB database.

**Manual curation of classification (1=likely to be a functional protein; 2=some minor red flags, but likely a functional protein). Supplemental Table 1 shows the full output of TransportTP analysis.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Solyc03g025240.1.1
Solyc03g025230.2.1
Solyc03g025190.2.1/MTP77
Solyc06g036130.2.1
VvAM1
VvAM2
VvAM3
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PtMATE
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At4g25640.1AtDTX35/FFT
MtMATE2
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Solyc10g051130.1.1
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Solyc05g013410.2.1
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Solyc12g019320.1.1
Solyc05g013450.2.1
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At1g12960.1AtDTX31/RHS2
At1g12950.1AtDTX29
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At1g33080.1AtDTX23
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MdMATE2
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MtMATE1
VvAnthoMATE
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NtMATE1
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At1g61890.1AtDTX37
At4g21900.1AtDTX38
At4g21910.1AtDTX39
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Solyc04g009080.2.1
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FIGURE 1. Phylogeny of MATE transporters in the tomato genome. All protein sequences of MATE transporters identified in the tomato genome (67 sequences), along the complete set in *Arabidopsis thaliana* (56 sequences) and other MATE transporters functionally characterized in other plant species (33 sequences). The analysis was conducted in MEGA7 [83] using Maximum Likelihood method with 1,000 bootstraps. Branches are drawn to scale in the number of substitutions per site. Notice the five clear clades encompassing MATE with distinct functional properties

The tree pattern obtained with five clades for the transport of potentially distinct substrates is congruent with previous studies [16, 43]. Clade 1 (blue) contains 24 tomato MATE transporters along with ROOT HAIR-SPECIFIC 2 (RHS2/DTX31) and FLOWER FLAVONOID TRANSPORTER (FFT/DTX35), from Arabidopsis; VvAM1 and VvAM3 from grapevine; PtMATE from poplar; and MtMATE2 from *Medicago truncatula*. The previously reported MTP77 (Solyc03g025190) from tomato [30] and NtJAT2 from tobacco [44] are also confined to clade 1. Many members of this clade have been functionally implicated in the transport of secondary metabolites. Importantly, FFT/DTX35 was considered as flavonoid transporter [45], but recent findings showed that, along with DTX33, it rather functions as a vacuolar chloride channel involved in cell turgescence during stomatal movements [46], root hair elongation, and pollen germination. MtMATE2 is a vacuolar anthocyanin transporter [47]; VvAM1 and VvAM3 are involved in anthocyanin transport to vacuoles in the grapevine [48]; NtJAT2 transports alkaloids, such as nicotine, into the vacuole of the tobacco leaf cells [44]. The substrate of the root-specific RHS2 [49] remains unknown. In tomato, since MTP77 is induced by the MYB transcription factor ANTHOCYANIN 1

(ANT1), it was presumed to be a permease that directs anthocyanins to the vacuole of leaf cells [30]. Although its full functional characterization is still lacking, but it might rather be a vacuolar chloride channel given the recent characterization of Arabidopsis DTX33 and DTX35 function [46]. Notwithstanding, the tomato MATEs in this clade are excellent candidates for mediating transport and cellular accumulation of alkaloids and phenolic compounds.

Clade 2 (purple) contains six tomato MATEs in addition to proteins known to mediate the transport of proanthocyanidins or anthocyanins to the vacuole, such as TT12 (DTX41) from Arabidopsis [28, 47], BrTT12 from turnip [50], MtMATE1 from *Medicago truncatula* [27], MdMATE1 and MdMATE2 from *Malus domestica* (apple) [51] as well as VvMATE1 and VvanthoMATE from grapevine [29, 52]. Also in this clade is SbMATE2 from sorghum, which transports hydroxynitrile glucosides (e.g., dhurrin)[53]. Importantly, among the tomato MATE proteins present in this group, Solyc12g006360 seems to be the best candidate for vacuolar sequestration of anthocyanins due to its close relation with characterized transporters. This result is significant, given the recent interest in breeding vegetables for high-nutrient density [11].

Clade 3 (green) contains 21 tomato MATEs along with only three that have been functionally characterized. NtJAT1 from tobacco is a jasmonate-inducible alkaloid carrier expressed in stem, roots, and leaves [54]. In coordination with NtJAT2, NtJAT1 plays a role in the vacuolar sequestration of alkaloids in tobacco (e.g., nicotine, anabasin, hyoscyamine, and berberine) [44, 54]. Interestingly, Solyc02g091070 and Solyc02g091080 show a close phylogenetic relationship to NtJAT1, indicating they may transport alkaloids in tomato. In Arabidopsis, ALF5/DTX19 is expressed in root epidermal cells and necessary for protecting roots from toxic compounds in the soil [55]. AtDTX18 is responsible for the secretion of the coumaroylagmatine and other hydroxycinnamic acid amides in response to *Phytophthora infestans* colonization [56]. From the few genes functionally characterized in the group, it is possible they transport toxic compounds to specific parts of the plant as a component of plant defense mechanisms.

Clade 4 (brown) consists of eleven tomato MATEs and six functional characterized transporters from other species. In Arabidopsis, ADS5/DTX47 participates in plant immune response by transporting salicylic acid upon induction by biotic stress [24]. On the other hand,

AtDTX51/ADP1/ADS1 inhibits SA accumulation [23]. The Golgi-localized BUSH AND CHLOROTIC DWARF 1 (BCD1/ZRIZ1/ZRZ/ABS4/ZF14/AtDTX48) is expressed in flowers, shoots, and the hypocotyl. It plays a role in Fe homeostasis, including during organ initiation and development [57, 58]. MtMATE55 was experimentally confirmed to play a similar role in the model legume, *Medicago truncatula* [22] further supporting the usefulness of phylogeny to predict function in the MATE family of distantly related plant species. ABNORMAL SHOOT 3-LIKE 1 (ABS3L1/DTX50) and ABS3L2/DTX52 are both implicated in the negative regulation of hypocotyl cell elongation [59]. RESISTANT TO HIGH CO_2 1 (RHC1/DTX56) is localized to the plasma membrane and participates in responses to increased CO_2 and stomatal closure by repression of HIGH LEAF TEMPERATURE 1 (HT1) and OPEN STOMATA 1 (OST1) protein kinases that link this MATE transporter to CO_2 signaling through bicarbonate sensing [60]. Overall, the MATE transporters in this group are related to Fe homeostasis and the transport of signaling molecules involved in diverse mechanisms of plant defense, growth, and development.

At last, clade 5 (pink) contains five tomato MATEs. The functionally characterize members of this group mediate citrate efflux and participate either in Fe uptake and metal homeostasis or Al^{3+} tolerance mechanisms. FERRIC REDUCTASE DEFECTIVE 3 (FRD3/DTX43) is essential for Zn^{2+} tolerance in Arabidopsis by regulating Fe homeostasis [61]. Likewise, GmFRD3a and GmFRD3b are induced by Fe deficiency in soybean [62]. In proteoid roots of the legume *Lupinus albus*, LaMATE is induced under P deficiency conditions [63]. Importantly, functional analysis of MATE of this group in Arabidopsis and cereals led to the development of useful genetic markers for improved crop tolerance to Al^{3+} in acidic soils [64]. Members of this clade have also been functionally characterized as citrate effluxers in several cereals, such as wheat (TaMATE1B) [65], rye (ScFRDL1 and ScFRDL2) [66], barley (HvAACT1) [67], rice (OsFDL1 and OsFRDL4) [68], sorghum (SbMATE) [64, 69], and maize (ZmMATE1) [70], not to mention in dicot species, such as cabbage (BoMATE) [19], eucalyptus (EcMATE1) [71], and the MATEs from legume models, LjMATE1 from *Lotus japonicus* [72] and MtMATE66 and MtMATE69 from *Medicago truncatula* [22]. The three tomato transporters in the subgroup (Solyc01g008420,

Solyc01g087150, Solyc11g065820) may be significant to improve tolerance to acidic soils in Solanaceae crops.

Also, a clear subclade containing two MATEs from tomato and three from Arabidopsis can be found at the root of clade 5. The ENHANCED DISEASE SUSCEPTIBILITY (EDS5/SCORD3/SID1/DTX47) from Arabidopsis participates in salicylic acid (SA) signaling for disease resistance [73]. Surprisingly, EDS5 localizes at the chloroplast envelope of epidermal cells [32] and mediates the influx of SA from this organelle to the cytosol upon stress [31]. It is quite significant that this small subclade displays a contrasting functional role (transport direction, as an influxer) along with a distinct subcellular localization. Given that Solanaceae crops are often vulnerable to multiple diseases, these two tomato MATE genes could be important elements to breed more disease tolerant varieties and worthy of functional studies.

Tandem duplications and synteny of MATE transporters in the tomato genome

The expansion of the MATE family in plant genomes in relation to other kingdoms is quite remarkable [14, 74]. This may relate to the sessile lifestyle of plants, which calls for many secondary metabolites, and hence transporters carrying out efflux or vacuolar sequestration of toxic substances, in order to cope with all the biotic and abiotic stresses inherent to the environment [75]. Furthermore, in general, the presence of multiple paralogs in multigene families may also relate to the recurring polyploidization events of the angiosperm lineage, which generated gene duplicates that have often been retained in extant plant genomes [76]. Over time, these duplicates may have culminated in sub- or neofunctionalization, and subsequently, acquired new functions that might occasionally be retained, thus resulting in functional diversity and proliferation of genes derived from a common ancestor gene [77]. The identification of closely related paralogs in genomes is useful to discover potential gene redundancies, whereas identifying true orthologues between species can lead to the creation of hypotheses of common gene functions in other species.

In order to establish strong evidence of homology, we assessed the microsynteny within the tomato genome, as well as the syntenic block

conservation between the tomato and Arabidopsis genomes. Our analysis revealed 13 tandem duplication segments containing 33 MATE genes (on chromosomes 1, 2, 3, 4, 5, 7 and 10) (**Table 2**).

TABLE 2. In-tandem MATE gene duplicates in the *S. lycopersicum* genome.

Tandem Duplication Groups	Loci
Group 1	Solyc01g109310 Solyc01g109320
Group 2	Solyc02g080490 Solyc02g080480
Group 3	Solyc02g063260 Solyc02g063270
Group 4	Solyc02g091050 Solyc02g091070 Solyc02g091080
Group 5	Solyc03g025200 Solyc03g025210 Solyc03g025220 Solyc03g025230 Solyc03g025240 Solyc03g025250
Group 6	Solyc03g112250 Solyc03g112260
Group 7	Solyc03g118960 Solyc03g118970
Group 8	Solyc04g007530 Solyc04g007540

Group 9	Solyc04g074840 Solyc04g074850
Group 10	Solyc05g008500 Solyc05g008510
Group 11	Solyc05g013450 Solyc05g013460 Solyc05g013470
Group 12	Solyc07g006730 Solyc07g006740
Group 13	Solyc10g007360 Solyc10g007370 Solyc10g007380

In-tandem MATE duplicates comprise 55% of the gene family in the tomato genome, which supports the role of this evolutionary mechanism for the expansion of the gene family [16, 78]. In monocots, as well as dicots (including rosids and asterids), the degree of paralog fractionation corresponds to the functional category and not to the genetic proximity between species, that is, genes linked to metabolic functions tend to be present in fewer copies in relation to those involved in regulation and stimulus response [79], as is the case of the MATE family. In addition, tandem duplications may result in an intensification of gene expression. This fact has been observed in corn, which varieties with three identical,

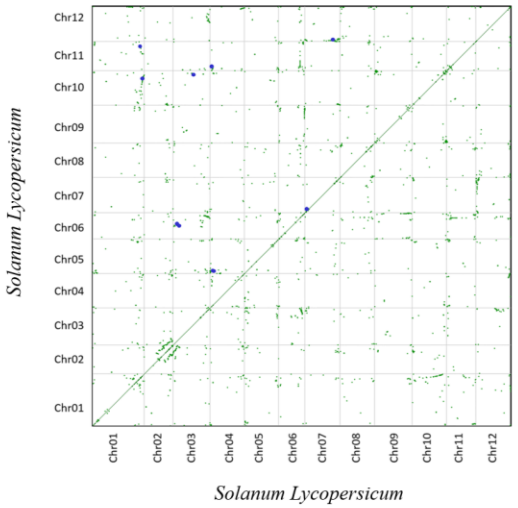
in-tandem MATE genes showed greater tolerance to Al^{3+} toxicity due to an increased expression of these genes [80].

Ten pairs of syntenic *MATE* paralogs were found within the tomato genome **(Figure 2A),** whereas seven ortholog pairs were identified in syntenic blocks between tomato and Arabidopsis (**Figure 2B**). In the phylogenetic context, the tomato paralogs belong to clade 1 (two syntenic pairs), clade 2 and 4 (three pairs each), thus establishing strong evidence of common ancestry (i.e., orthology) as well as allowing us to propose robust hypotheses of functional conservation between the *MATE* genes that have already been functionally characterized in Arabidopsis and their syntenic pairs in tomato, especially when the expression patterns are preserved over the course of evolution.

We also observed that, in some instances, duplicated tomato MATE genes showed conserved synteny with Arabidopsis genes (**Figure 2B**). Solyc03g025190/MTP77 is microsyntenic to Solyc06g036130 (paralog pair 3), and syntenic to At4g25640/AtDTX35/FFT (ortholog pair 3, **Figure 2B**). Based on this information, both of these tomato transporters may possibly function as vacuolar chloride channels involved cell turgescence

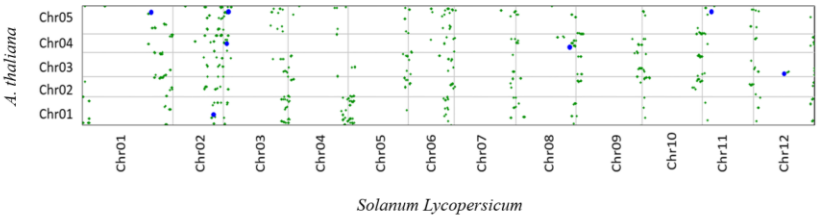
(clade 1, **Figure 1**). Solyc03g026230 is microsyntenic to Solyc06g035710 (paralog pair 4), and orthologous to At5g52050/AtDTX50/ABS3L2 (ortholog pair 4). AtDTX50 (clade 4) is implicated in plant development and growth by potentially inhibiting hypocotyl elongation, although its substrate is unknown [59]. Solyc12g005850 is microsyntenic to Solyc07g052380 (paralog pair 10), and syntenic to At3g21690/AtDTX40 (ortholog pair 7). They belong to clade 2 and are probably connected to the transport of anthocyanins or other flavonoids, since AtDTX41/TT12 [47], BrTT12 [50], VvAM1[48], and MtMATE1 [27] cluster together in this clade. At last, we noticed that the Solyc02g080480/Solyc02g080490 in-tandem pair duplicate on chromosome 2 is syntenic to At1g11670/AtDTX36 (ortholog pair 2) and, given their location in clade 2), they are probably connected to the transport of anthocyanins or other flavonoids.

A



Paralog Pairs	Loci
Pair 1	Solyc01g008420 Solyc11g065820
Pair2	Solyc01g110280 Solyc10g054100
Pair3	Solyc03g025190 Solyc06g036130
Pair4	Solyc03g026230 Solyc06g035710
Pair4	Solyc03g063730 Solyc10g080340
Pair5	Solyc04g007530 Solyc05g008510
Pair6	Solyc04g007540 Solyc05g008500
Pair7	Solyc04g009790 Solyc11g006410
Pair8	Solyc05g008500 Solyc04g007540
Pair9	Solyc07g006730 Solyc07g007490
Pair10	Solyc07g052380 Solyc12g005850

B



Syntenic Pairs	*Solanum lycopersicum*	Arabidopsis
Pair 1	Solyc01g066560	At5g49130/AtDTX55
Pair 2	Solyc02g080480	At1g11670/AtDTX3
Pair 3	Solyc03g025190 Solyc06g036130	At4g25640/AtDTX35
Pair 4	Solyc03g026230 Solyc06g035710	At5g52050/AtDTX50
Pair 5	Solyc08g079730	At4g22790/AtDTX56
Pair 6	Solyc11g010380	At5g65380/AtDTX28
Pair 7	Solyc12g005850	At3g21690/AtDTX40

FIGURE 2. Syntenic analyses of MATE genes in the tomato genome. (A) Identification of paralog pairs in microsyntenic blocks within the tomato

genome. Ten gene pairs were identified. (B) Synteny analysis between MATE transporters in the tomato and Arabidopsis thaliana genomes. Seven syntenic paralogs were found in this analysis. Blue dots were plotted according to gene coordinates within the respective chromosomes.

Therefore, we identified tomato MATE transporters potentially transporting flavonoids, alkaloids, and signaling molecules. Likely, their physiological functions have been conserved at least since the last common ancestor between these two species, which is estimated to have existed circa 150 million years ago [81].

Expression patterns of MATE genes in the tomato plant

The expression analysis of the 67 tomato MATE genes identified was performed using the TomExpress platform (http://gbf.toulouse.inra.fr/tomexpress/www/query.php). A heatmap of gene expression was generated with 19 representative samples in different organs. The genes are displayed according to their phylogenetic associations (**Figure 3**). While 33 tomato MATE genes are constitutively expressed in the dataset, 34 showed changes in their transcriptional activity.

Clade 1 (blue) contains tree genes with constitutive, high expression (Solyc10g081260; Solyc11g010380 and Solyc04g074850).

Solyc03g025240 has the lowest and most specific expression in the group, which, given its close phylogenetic relationship with NtJAT2 from tobacco (**Figure 1**), probably transports alkaloids in roots, flowers, and fruits in a very early developmental stage. A previous attempt to assign a function for Solyc10g081260 as a potential phenolics transporter took into account a <2-fold change of expression between M82 and an introgression line (IL7-3 from *S. pennellii*) [8]. Importantly, the study did not consider the ubiquitous, constitutive and high expression throughout the plant. Although our studies do not rule out this possibility, we propose that Solyc10g081260 is likely ought to transport alkaloids.

In contrast, only two tomato MATEs in clade 2 (purple) showed constitutive expression (Solyc02g080490 and Solyc12g005850). Other members of this clade show varied levels of transcriptional activity in diverse tissues. Possibly, these transporters may have an affinity for secondary metabolites that are not present in every organ of the plant, such as the transport of flavonoids to the vacuole.

In relation to the movement of defense compounds, clade 3 members (green) encompass Solyc02g063270, which is highly expressed

in all tissues studied. On the other hand, two members (Solyc03g11250, and Solyc03g112260) showed varying, low expression across tissues, while Solyc10g007380 was highly expressed in flowers, and some expression during the early stages of fruit development.

Of the eleven transporters in clade 4 (brown), four showed constitutive expression, although at mid-to-low levels in most tissues analyzed. Given the close phylogenetic relationship (**Figure 1**) and similar expression pattern (**Figure 3**), it is tempting to suggest the same potential role of Solyc08g080310 as that assigned for the Arabidopsis BCD1/DTX48, which is expressed in flowers and vegetative shoots, and plays a role in Fe nutrition during organ initiation and development [16, 57, 58].

Unlike other clades, all MATE genes in clade 5 (pink) were constitutively expressed, with varying transcriptional intensities. Members of this group have been related to the transport of citrate and detoxification of Al^{3+} in roots as well as Fe translocation throughout the plant. The constitutive expression patterns of clade 5 members suggest they participate in physiological mechanisms throughout the whole plant.

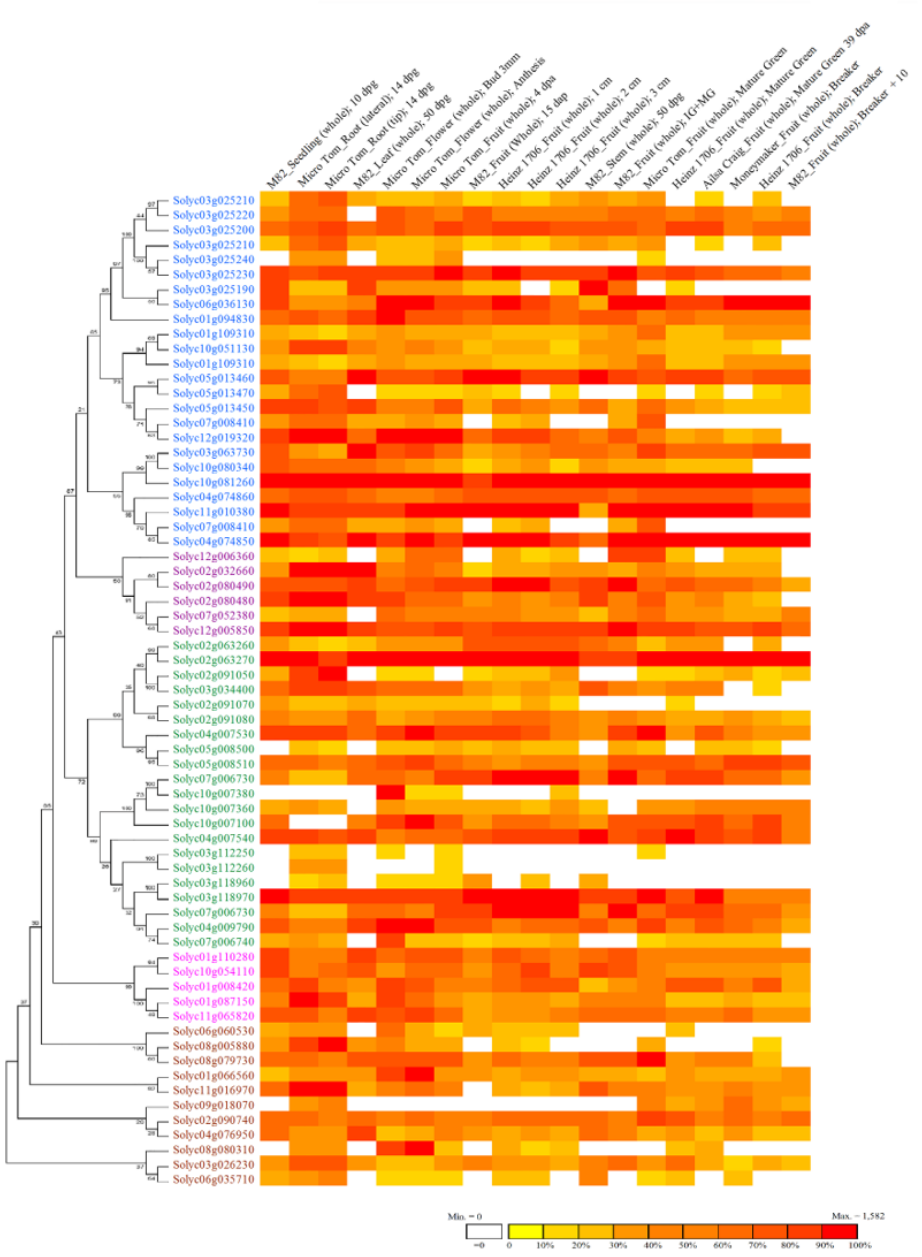

M82_Seedling (whole); 10 dpg
Micro Tom_Root (lateral); 14 dpg
Micro Tom_Root (tip); 14 dpg
M82_Leaf (whole); 50 dpg
Micro Tom_Flower (whole); Bud 5mm
Micro Tom_Flower (whole); Anthesis
M82_Fruit (Whole); 4 dpa
Heinz 1706_Fruit (whole); 15 dap
Heinz 1706_Fruit (whole); 1 cm
Heinz 1706_Fruit (whole); 2 cm
M82_Stem (whole); 3 cm
M82_Fruit (whole); 50 dpg
Micro Tom_Fruit (whole); IG+MG
Heinz 1706_Fruit (whole); Mature Green
Alisa Craig_Fruit (whole); Mature Green
Moneymaker_Fruit (whole); Mature Green 39 dpa
Heinz 1706_Fruit (whole); Breaker
M82_Fruit (whole); Breaker + 10
Solyc03g025210
Solyc03g025220
Solyc03g025200
Solyc03g025210
Solyc03g025240
Solyc03g025230
Solyc03g025190
Solyc06g036130
Solyc01g094830
Solyc01g109310
Solyc10g051130
Solyc01g109310
Solyc05g013460
Solyc05g013470
Solyc05g013450
Solyc07g008410
Solyc12g019320
Solyc03g063730
Solyc10g080340
Solyc10g081260
Solyc04g074860
Solyc11g010380
Solyc07g008410
Solyc04g074850
Solyc12g006360
Solyc02g032660
Solyc02g080490
Solyc02g080480
Solyc07g052380
Solyc12g005850
Solyc02g063260
Solyc02g063270
Solyc02g091050
Solyc03g034400
Solyc02g091070
Solyc02g091080
Solyc04g007530
Solyc05g008500
Solyc05g008510
Solyc07g006730
Solyc10g007380
Solyc10g007360
Solyc10g007100
Solyc04g007540
Solyc03g112250
Solyc03g112260
Solyc03g118960
Solyc03g118970
Solyc07g006730
Solyc04g009790
Solyc07g006740
Solyc01g110280
Solyc10g054110
Solyc01g008420
Solyc01g087150
Solyc11g065820
Solyc06g060530
Solyc08g005880
Solyc08g079730
Solyc01g066560
Solyc11g016970
Solyc09g018070
Solyc02g090740
Solyc04g076950
Solyc08g080310
Solyc03g026230
Solyc06g035710
Min. = 0
Max. = 1,582
=0
0
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%

FIGURE 3. Expression profiling of tomato MATE transporters. Selected RNA-Seq samples were analyzed using TomExpress tool, and the genes were ordered according to their phylogenetic associations.

In conclusion, the global analysis of 67 MATE genes identified in the tomato genome revealed potential functional relationships with transporters characterized in other plant species, as well as potentially interesting targets for functional studies. Such analyses are crucial not only to better understand secondary metabolism in tomato, but also to identify key genes for breeding purposes.

Material and Methods

Identification of MATE transporters in the tomato genome. The full protein set of tomato (ITAG2.4 release: ftp://ftp.solgenomics.net/tomato_genome/annotation/ITAG2.4_release/ITAG2.4_proteins.fasta) was submitted to the TransportTP transporter prediction tool (http://bioinfo3.noble.org/transporter/) [42] for identification of membrane transporters followed by classification into transporter families according to the Transporter Classification system (TCDB, http://www.tcdb.org) [82]. Manual curation for the MATE family followed as previously described [41].

Phylogenetic analyses. The evolutionary analysis was conducted in MEGA7 [83] and involved 156 full-length MATE amino acid sequences from tomato (67 sequences), Arabidopsis (56 sequences) and those functionally characterized from other plant species (33 sequences). The phylogenetic analyses were inferred by the Maximum Likelihood method with a bootstrap of 1,000 replicates, based on the JTT matrix-based model [84]. The initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated with the JTT model, and the topology was selected with superior log-likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

Analyses of synteny. We used the CoGe comparative genomic toolkit (https://genomevolution.org/coge/) [85] to identify in-tandem MATE duplications in tomato, syntenic genes within its genome, as well as syntenic (collinear) gene blocks between tomato and *Arabidopsis thaliana*.

Transcriptional profiling of MATE transporters. Relative expression of the 67 genes of tomato MATE family was carried out using the tool TomExpress (http://gbf.toulouse.inra.fr/tomexpress/www/query.php), as previously described [86].

Acknowledgments

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES) from the Brazilian Ministry of Education for the full scholarship provided to A.L.S. and S.C.S.

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL TABLE S1. TransportTP output for the MATE gene family in the *S. lycopersicum* genome.

ARTIGO 5: Identificação de um potencial membro da família mate (*multidrug and toxic compound extrusion*) responsável pelo transporte de antocianina em tomate

A ser submetido para PLOS ONE JOURNAL

Identificação de um potencial membro da família mate (*multidrug and toxic compound extrusion*) responsável pelo transporte de antocianina em tomate

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RESUMO

Os transportadores de membrana MATE (*Multidrug and Toxic Compound Extrusion*) abrangem uma família de transportadores secundários presentes em bactérias, fungos, mamíferos e plantas. Espécies da família Solanaceae são conhecidas pela grande quantidade de metabólitos secundários que produzem, incluindo alcalóides, terpenóides e compostos fenólicos. Dentre os compostos fenólicos, o consumo dietético das antocianinas se destaca devido seus benefícios à saúde humana. Além das vias de biossíntese, uma compreensão abrangente do metabolismo secundário também deve levar em conta o transporte e a compartimentação subcelular de substâncias. Neste sentido, é de grande importância mais pesquisas com o intuito de reconhecimento e caracterização de transportadores membros da família MATE. A maioria dos transportadores MATE foram caracterizados para mediar o transporte de metabolitos primários e secundários utilizando gradiente eletroquímico de H^+ ou Na^+ através da membrana. O objetivo com esse trabalho foi identificar e testar o padrão de expressão de um gene MATE potencialmente correlacionado com o acúmulo de antocianina em frutos de tomateiro (*Aft/atv/hp2)* por meio de análise filogenética e ensaio de qRT-PCR. Foram previamentes identificados 67 genes que codificam para transportadores MATE. Por meio de análise filogenética foi observado que 20 dos 67 genes podem estar relacionados com o transporte de antocianina, dentre eles, o gene Solyc12g006360 é o melhor candidato à função de transporte de antocianinas para o interior do vacúolo em tomates devido sua proximidade com outros genes já caracterizados com a mesma função. Ademais, por meio de um ensaio de qRT-PCR foi observado que a expressão relativa de Solyc12g006360 é maior em tecidos que acumulam antocianina em relação aos tecidos que não a acumulam.

Palavras-chave: *Solanum lycopersicum*, Antocianina, Tomate roxo.

ABSTRACT

MATE (*Multidrug and Toxic Compound Extrusion*) membrane carriers comprise a family of secondary carriers present in bacteria, fungi, mammals and plants. Species of the family Solanaceae are known for the large amount of secondary metabolites they produce, including alkaloids, terpenoids and phenolic compounds. Among phenolic compounds, dietary intake of anthocyanins stands out due to its benefits to human health. In addition to biosynthetic pathways, a comprehensive understanding of secondary metabolism should also take into account subcellular transport and compartmentalization of substances. In this sense, it is of great importance more researches with the intention of recognition and characterization of transporters members of the MATE family. Most MATE transporters were characterized to mediate the transport of primary and secondary metabolites using electrochemical gradient of H + or Na + across the membrane. The objective of this work was to identify and test the expression pattern of a MATE gene potentially correlated with anthocyanin accumulation in tomato fruits (*Aft/atv/hp2*) by phylogenetic analysis and QRT-PCR assay. 67 genes encoding MATE transporters were previously identified. By means of phylogenetic analysis it was observed that 20 of the 67 genes may be related to the transport of anthocyanin, among them, the gene Solyc12g006360 is the best candidate for the function of transport of anthocyanins to the interior of the vacuole in tomatoes due to its proximity with other genes Already characterized with the same function. In addition, by means of an QRT-PCR assay it was observed that the relative expression of Solyc12g006360 is higher in anthocyanin accumulating tissues compared to tissues that do not accumulate it.

wq: *Solanum lycopersicum*, Anthocyanin, Purple tomato.

INTRODUÇÃO

A família gênica *Multidrug and toxic compound extrusion* (MATE) de transportadores de membrana está universalmente presente em todos os domínios de organismos. MATE é uma família de transportadores que exportam produtos xenobióticos usando o gradiente eletroquímico de H^+ ou Na^+ através de membranas celulares [1]. Por se tratarem de proteínas que exercem o transporte de diversos substratos, MATEs podem ser alvos de programas de melhoramento genético para o aprimoramento de características de interesse. Nesse sentido, mais pesquisas a respeito da caracterização funcional dessas proteínas são necessárias para o melhor entendimento sobre a fisiologia e metabolismo das plantas.

Vários trabalhos já demostraram funções importantes dos transportadores MATE em bactérias, leveduras, mamíferos e plantas [1-5]. Em tomate (*Solanum lycopersicum*), foram identificados 67 membros da família MATE, previamente descritos anteriormente. Espécies da família Solanaceae são conhecidas pela grande quantidade de metabólitos secundários que produzem, incluindo alcalóides, terpenóides e compostos fenólicos. Dentre os compostos fenólicos, as antocianinas têm se destacado devido suas atividades benéficas à saúde [6-8]. Já se tem relatados de MATEs responsáveis pelo transporte de antocianina como por exemplo em *Medicago truncatula*, *Arabidopsis thaliana*, *Vitis vinifera* e *Malus domestica*. Entretanto, pouco ainda se sabe sobre a proteína MATE responsável pelo transporte de antocianina em tomates (*Solanum lycopersicum*).

Nesse sentido, o objetivo com esse trabalho foi identificar e testar o padrão de expressão de um gene MATE potencialmente correlacionado com o acúmulo de antocianina em frutos de tomateiro (*Aft/atv/hp2)* por meio de análise filogenética e ensaio de qRT-PCR.

MATERIAL E MÉTODOS

Análise filogenética

Análise filogenética prévia com todos membros da família MATE de transportadores de membrana em *Solanum lycopersicum,* (67 membros) foi conduzida juntamente com 56 sequências de Arabidopsis, bem como 34 transportadores MATE que foram funcionalmente caracterizados em outras espécies de plantas, totalizando 157 membros. Por meio desta análise, uma árvore filogenética subdividida em 5 clados foi gerada. Desses, foram selecionados os membros de dois clados (totalizando 66 membros de várias espécies vegetais), que são membros representantes da família MATE relacionados por transportar alcalóides e flavonóides, incluindo antocianinas. Neste trabalho, uma nova análise filogenética foi realizada e uma árvore foi gerada com os membros selecionados (Figura 1). A análise filogenética foi realizada pelo programa MEGA7 [78] e inferidas pelo "*Maximum Likelihood Method*" com bootstrap de 1.000 repetições e modelo em matrix JTT [79]. As árvores foram desenhadas em escala, com comprimento de ramos representando substituições por local.

Quantificação relativa da expressão dos genes

Coleta do material vegetal

Plantas do tipo selvagem (Micro-Tom) e mutante isogênico a MT (*Aft/atv/hp2*) foram cultivadas em casa de vegetação sob condições normais de cultivo. Frutos de três plantas de cada genótipo foram coletados na fase madura. Cada fruto foi dissecado em epicarpo e mesocarpo, congelados imediatamente em nitrogênio líquido e armazenados em ultrafreezer a -80^{o}C para análises posteriores.

Extração de RNA total, tratamento com DNase I e síntese de cDNA

RNA total foi extraído separadamente das diferentes partes do fruto com reagente TRIzol® (Life Technologies), com protocolo do fabricante para extração de RNA total. Um micrograma do RNA foi tratado com DNase I (TURBO DNase, Life Technologies) e usado para síntese de cDNA com primers oligo(dT) e o kit SuperScript III reverse transcriptase (Life Technologies).

Expressão dos genes por qRT-PCR

Para a análise de expressão do gene selecionado foi feita em aparelho ABI PRISM 7500 Real-Time PCR (*Applied Biosystems*), com método de detecção via *SYBR Green* em triplicatas biológicas. Os primers utilizados estão mostrados na Tabela 1 e suas respectivas eficiências foram determinadas pelo software LinRegPCR [9].

No ensaio de expressão, em cada reação utilizou-se 1 µl de cDNA (diluído 1:10), 1,5 µM de primer *forward/reverse* mix (0,75 µM cada) e 2,5 µL de 2X *MasterMix SYBR Green* (*Applied Biosystems*), totalizando um volume final de 5 µL. As amostras foram pipetadas em triplicatas técnicas, e um controle sem cDNA (NTC) foi incluído para cada par de primers. Os resultados foram normalizados usando CTs (linhas de base) obtidos pela expressão do gene de referência *β-tubulina* (*Solyc04g081490*). O CT foi determinado pelo número de ciclos no qual a fluorescência gerada dentro de uma reação cruza o CT. A expressão relativa foi analisada pelo método Pfaffl (Pfaffl 2001).

As condições térmicas da reação foram: 2 minutos a 50°C e 10 minutos a 95°C para iniciação, seguidos por 40 ciclos de 15 segundos a 95°C e 1 minuto a 60°C, e finalizando-se com 15 minutos a 95°C. Ao fim da ciclagem, uma curva de desnaturação de 60-95°C (X segundos por grau de aquecimento) mostrou a especificidade da reação de PCR. Os dados foram coletados, exportados pelo

programa *7500 Fast Software* (Versão 2.1) e analisados em planilha Excel (Microsoft).

Tabela 1. Primers utilizados para amplificação dos fragmentos.

housekeeper *(ß-tubulina)* (*Solyc04g081490*) 109pb	**Tom_bTub-qPCR-fw**	**Tom_bTub-qPCR-rv**
	AAGATGGCATCCACGGTTTGT	ACCAATGCAAGAAAGCCTTG
MATE (*Solyc12g006360*) 84pb	**Solyc12g006360-MATE-Fw**	**Solyc12g006360-MATE-Rv**
	TGACTCCATTACTTGCCATTTC	GAACGGTAGGTGAAGGTTG

Análise *in silico* da expressão

A análise de expressão i*n silico* foi realizada na plataforma TomExpress (http://gbf.toulouse.inra.fr/tomexpress/www/query.php) com dados de tomates selvagens (*Solanum lycopersicum*), onde foi gerado o perfil de expressão da proteína *Solyc12g006360* em diferentes tecidos do tomateiro (Figura 2).

RESULTADOS E DISCUSSÃO

Análise filogenética

Por meio da análise filogenética realizada utilizando os 66 transporadores MATE dos clados 1 e 2 associados ao transporte de antocianinas, obtivemos um agrupamento similar à análise prévia (Capítulo 4, Figura 1), onde o clado azul é formado por 46 proteínas MATEs (incluindo 23 de tomateiro), muitas das quais foram caracterizadas como transportadores de flavonoides e alcaloides. O clado roxo é formado por 20 proteínas, incluindo 6 de tomateiro.

As proteínas MATE já relatadas como transportadoras de antocianina aparecem agrupadas tanto no clado roxo (MdMATE1, MdMATE2, VvMATE1, VvanthoMATE, MtMATE1, AtDTX41/TT12 e BrTT12), enquanto outras estão localizadas no clado azul, relacionado ao transporte de flavonóides (VvAM1-3, PtMATE e MtMATE2). A formação de mais de um grupo de proteínas MATE relacionadas com o transporte de antocianinas também podem ser observado em outros trabalhos como por exemplo em sorgo [10], em Medicago [11] e videira [12].

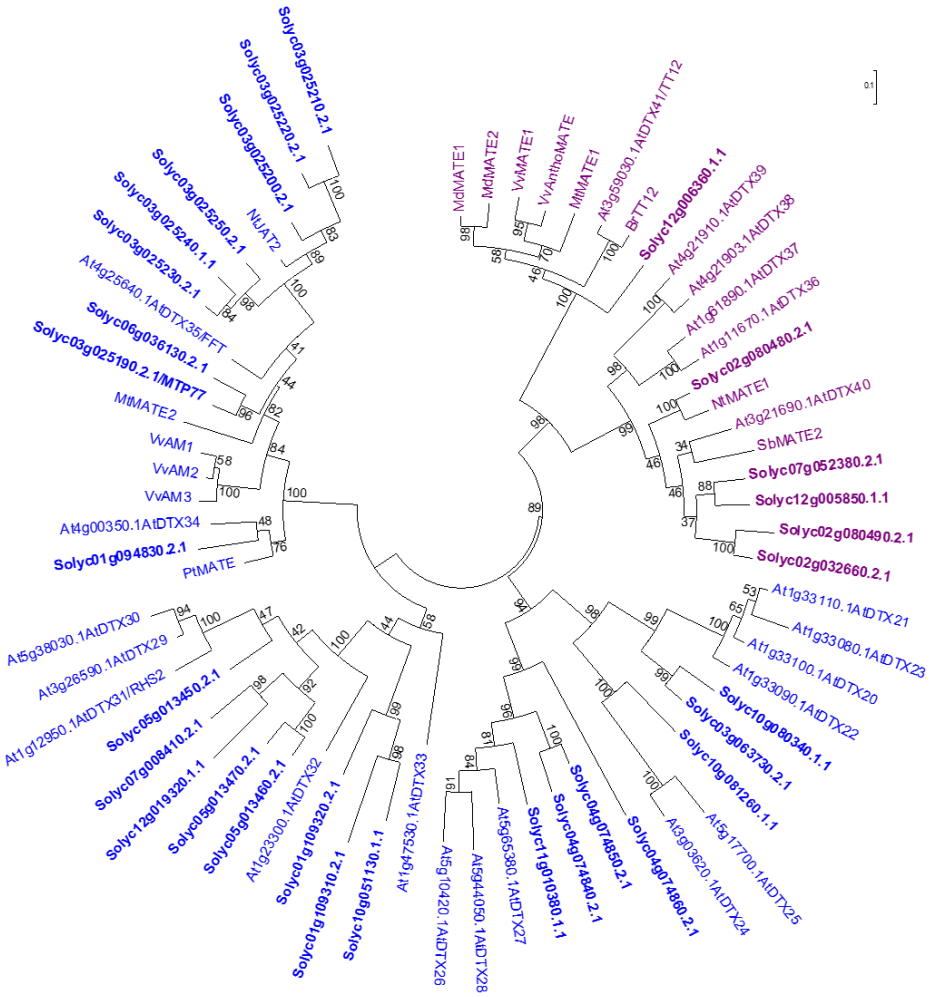

Figura 1. Análise filogenética dos 66 membros agrupados nos clados 1 e 2 representados no Capítulo 4 (Figura1). A análise de filogenia foi feita por método idêntico ao descrito no Capítulo 4.

No clado roxo, destaca-se um subclado onde estão presentes membros MATE que já foram caracterizados funcionalmente como transportadores de antocianina: MdMATE1 e MdMATE2 em maçã; VvMATE1 e VvAnthoMATE em videira; DTX34/TT12 de Arabidopsis e BrTT12 de nabo (Figura 1). Neste subclado também se encontra um único membro ainda não caracterizado:

Solyc12g006360, o que indica ser o melhor candidato à função de transporte de antocianinas em céluas de tomateiro. *Solyc12g006360* codifica uma proteína de 512 resíduos de aminoácidos e 12 domínios transmembrana, o que indica um produto gênico funcional em acordo com as características gerais dos transportadores MATE.

Para maiores evidências da função putativa de *Solyc12g006360* no transporte de antocianina em tomates, foi realizado um ensaio de qRT-PCR afim de analisar o perfil de expressão desse gene em tecidos pigmentados e não pigmentados de frutos roxos de tomateiro (MT-*Aft/atv/hp2*).

Análise de expressão por qRT-PCR e *in silico*

O padrão de expressão de *Solyc12g006360* é condizente com distribuição dos tecidos dos genótipos analisados. O gene apresenta alta expressão no epicarpo dos frutos em relação ao mesocarpo. Além disso, *Solyc12g006360* apresentou maior expressão no epicarpo de tomates *Aft/atv/hp2*, o qual possui acúmulo de antocianina. A menor expressão foi observada no mesocarpo de frutos selvagens (Micro-Tom). A diferença de expressão foi maior no epicarpo de tomates *Aft/atv/hp2* em relação ao mesocarpo de tomates selvagens, que obtiveram menor expressão (Figura 2).

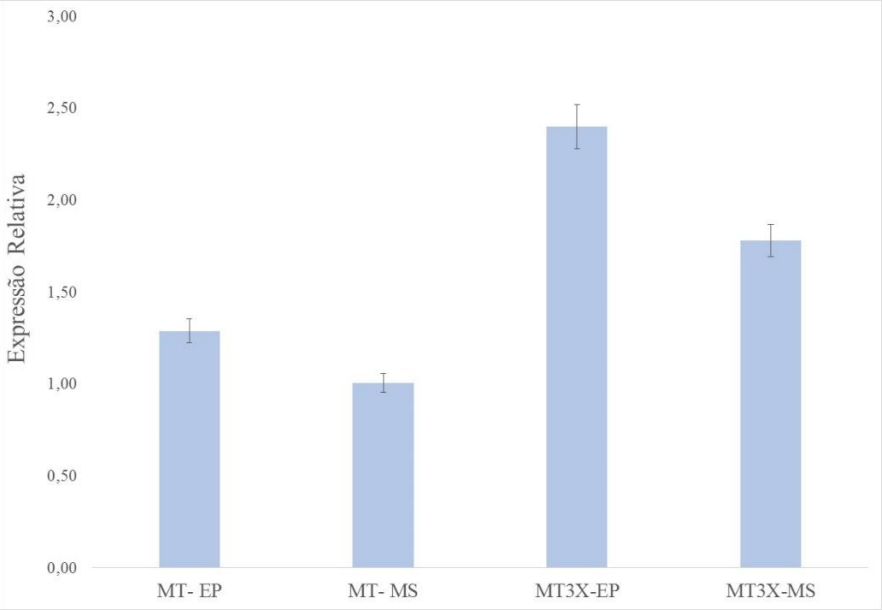

Figura 2. Perfil de expressão relativa de *Solyc12g006360* em mesocarpo e epicarpo de tomateiros mutantes MT -*Aft/atv/hp2* e em tipo selvagem (MT). MT: Micro-Tom selvagem; MT3X: MT-*Aft/atv/hp2*; EP: Epicarpo; MS: Mesocarpo. A expressão foi normalizada com a expressão de β-tubulina e relativa à expressão em mesocarpo de frutos selvagens.

Mesmo em tecidos que não acumulam antocianina substancialmente, como por exemplo no caso do mesocarpo de tomates selvagens, pode-se observar expressão de *Solyc12g006360*. Dessa forma, é possível que *Solyc12g006360* transcreva um transportador não exclusivo de antocianina, mas talvez, também outros flavonóides, devido à sua localização na árvore filogenética.

É interessante notar que proteínas MATEs responsáveis por transportar antocianinas parecem não transportá-las exclusivamente. Em mirtilo, MATEs ligados ao transporte de antocianina também são expressos em tecidos não pigmentados, como botões florais, flores e frutos verdes [13]. Da mesma forma, a expressão de algumas proteínas já caracterizadas localizadas no mesmo subclado que *Solyc12g006360* também possuem expressão em tecidos não pigmentados, como por exemplo no caso de VvMATE, expresso em tecidos

reprodutivos e frutos pequenos [12], e MdMATE1 e MdMATE2 em maçã, que apresentam expressão não só no epicarpo ciânico, mas também no mesocarpo e folhas aciânicos [14].

Ademais, por meio da análise *in silico*, observa-se que *Solyc12g006360* em plantas selvagens (*S. lycopersicum*) é expresso em botões florais, flores e frutos (Figura 3), enquanto não é expresso em folhas. Esse dado condiz com a expressão relativa por meio de qRT-PCR, onde também foi observado expressão do gene em tomates vermelhos, enquanto o gene não é expresso em folhas de tomateiro cv. MT. Essa análise reforça a hipótese de que antocianina não deve ser o único substrato de *Solyc12g006360*.

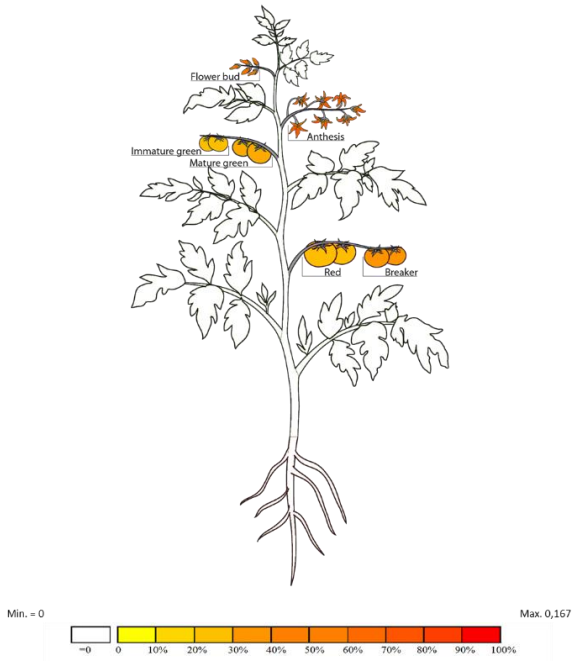

Figura 3. Expressão de *Solyc12g006360* em tecidos de tomateiro. Figura gerada na plataforma TomExpress. (http://gbf.toulouse.inra.fr/tomexpress/www/query.php). Tecidos pigmentados representam o nível de expressão de acordo com o indicado na barra de cores.

CONCLUSÃO

Solyc12g006360 transcreve para uma proteína potencialmente funcional de transportador MATE, provavelmente responsável pelo transporte de antocianina, mas não exclusivo de antocianina, mas também de outros compostos flavonóides. É provável que o acúmulo de antocianinas em vacúolos de células de tomateiro seja feito redundantemente por diversos transportadores de membrana, incluindo diversos da família MATE.

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