



Rootstock mediates transcriptional regulation of citrulline metabolism in grafted watermelon

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(With 5 figures)

Abstract

Citrulline is a non-essential amino acid, involved in key biological functions in plants and humans. Rootstocks have a major impact on citrulline accumulation in grafted watermelon. Information regarding rootstock induced changes in citrulline metabolism is elusive. To understand the regulatory mechanism, parallel changes in the expression profiles of citrulline metabolic genes and citrulline content of watermelon were monitored during the development of self-rooted watermelon and watermelon grafted onto pumpkin, wild and bottle gourd rootstocks. Results demonstrated that rootstocks regulated the expression profiles in different ways to influence the citrulline content. *GAT*, *NAGPR*, *ASS3*, *ASS2* and *ASL2* showed the negative correlation with citrulline content in pumpkin grafted watermelon. Pumpkin rootstock promoted the citrulline content by high down-regulation and synergistic effect of *ASS2*, *ASS3*, *ASL1* and *ASL2* genes. In wild grafted watermelon, citrulline was accumulated as a result of down regulation of *GAT*, *NAGS* and *ASL2* genes, which showed an inverse correlation with citrulline. In gourd grafted watermelon, changes in citrulline content were observed to be linked with lower expressions of *GAT*, *NAGK*, *ASS2*, *ASS3*, *ASL1* and *ARG* which were negatively correlated with citrulline content. Our study will provide the basis to understand the molecular mechanism of citrulline accumulation in various rootstocks.

Keywords: *Citrullus lanatus*, citrulline, gene expression, grafting, qRT-PCR.

Mediação do porta-enxerto na regulação transcricional do metabolismo da citrulina na melancia enxertada

Resumo

A citrulina é um aminoácido não essencial, envolvida em importantes funções biológicas de plantas e seres humanos. Os porta-enxertos têm um grande impacto no acúmulo de citrulina na melancia enxertada. Informações sobre alterações induzidas por porta-enxertos no metabolismo da citrulina ainda não foram descritas. Para entender o mecanismo regulatório, foram monitoradas mudanças paralelas nos perfis de expressão dos genes metabólicos de citrulina e no teor de citrulina da melancia durante o desenvolvimento da melancia e da melancia enxertada em porta-enxertos de abóbora, silvestre e cabaça. Os resultados demonstraram que o porta-enxerto regulou os perfis de expressão de diferentes maneiras para influenciar no conteúdo de citrulina. *GAT*, *NAGPR*, *ASS3*, *ASS2* e *ASL2* apresentaram correlação negativa com o teor de citrulina em melancia enxertada de abóbora. O porta-enxerto de abóbora promoveu o conteúdo de citrulina por meio de baixa regulação e efeito sinérgico de duas famílias de genes *ASS* e *ASL*. Na melancia enxertada, a acumulação de citrulina resultou na regulação negativa de *GAT*, *NAGS* e *ASL2*, que mostraram uma correlação inversa com a citrulina. Na melancia enxertada, observou-se que as alterações no conteúdo de citrulina foram associadas a menores expressões de *GAT*, *NAGK*, *ASS2*, *ASS3*, *ASL1* e *ARG*, que foram negativamente correlacionadas com o conteúdo de citrulina. Esses resultados fornecem a base para identificar o mecanismo molecular do acúmulo de citrulina em vários porta-enxertos.

Palavras-chave: *Citrullus lanatus*, citrulina, expressão gênica, enxerto, qRT-PCR.

1. Introduction

Watermelon [*Citrullus lanatus* Matsum. & Nakai var. *lanatus*] is an important member of the Cucurbitaceae family, belongs to the genus xerophyte originated in the desert of southern Africa (Erickson et al., 2005). According to 'FAO',

approximately 90 million tons of watermelon is produced all over the world annually. Watermelon is ranked among the 20th most important crop of the world, watermelon shares 7% cultivated land among other vegetables. China

is the largest producer of watermelon and ranks second in its production, of which 20% comes from grafted plants. Watermelon with large fruit size provides an enormous amount of health-promoting nutrients like vitamins, minerals, fiber, antioxidants, lycopene, sugars, and health-promoting amino acids such as citrulline, arginine, and glutathione which are required in our daily intake (Collins et al., 2007; Hayashi et al., 2005; Perkins-Veazie et al., 2006).

Citrulline is a nonessential amino acid found in substantial amount in watermelon. Watermelon is the largest source of natural citrulline to the human diet. It was ignored for many years until the discovery of its antioxidant characteristics and earned great importance in the scientific world. Citrulline is involved in major biological functions in plants as well as in humans. Citrulline is not involved in protein synthesis due to absence of tRNA but have a role in a post-transcriptional modification, which helps the plant to combat against drought stress. Additionally, it is involved in nitrogen translocation which makes it a major intermediate of the urea cycle (Joshi and Fernie, 2017). Citrulline has pleiotropic effects: it has a central role in the cardiovascular system (Romero et al., 2006) act as a regulator of immunity (Norris et al., 1995) and play a key role in the regulation of nitrogen homeostasis (Osowska et al., 2004, 2006).

In humans, citrulline has been suggested to cure problems related to sex stamina, pregnancy and muscles (Breuillard et al., 2015; Lassala et al., 2009).

Moreover, its role as a hydroxyl radical scavenger has been reported under drought and strong light conditions (Inukai and Suyama, 1966; Mapelli et al., 2001). Arginine is a downstream product of citrulline, genes of arginine pathway have massively been reviewed in recent years (Slocum, 2005; Winter et al., 2015). Series of enzymes are involved in the production of arginine from ornithine in a linear pathway (Micallef and Shelp, 1989; Slocum, 2005). Genes encoding enzymes of arginine pathway have been well characterized in animals, fungi as well as in prokaryotes but in plants biochemical characterization is partial (Caldovic and Tuchman, 2003; Condurso et al., 2012; Cunin et al., 1986; Davis, 1986; Fallik and Ilic, 2014; Shargool et al., 1988). Formation of citrulline starts with an acetylation of glutamate (Shargool et al., 1988) followed by phosphorylation, reduction, and transamination yielding ornithine in cyclic pathway by several enzymes that include *GAT* (glutamate N-acetyltransferase), *NAGS* (N-acetyl glutamate synthase), *NAGK* (N-acetyl glutamate kinase), *NAGPR* (N-acetylglutamate 5-phosphate reductase) and *NAOT* (N-acetylornithine transaminase). In the first step, *NAGS* uses acetyl-coenzyme A (Acetyl-CoA) to transfer an acetyl moiety to glutamate forming N-acetylglutamate (Shargool et al., 1988) N-acetylglutamate is then phosphorylated at the C5 position by *NAGK*. In the next step, the formation of N-acetylglutamate-5-semialdehyde (*NAcGSA*), is catalyzed by *NAGPR*. In the fourth step, the second glutamate delivers another amino group to a molecule of N-acetylglutamate-5-semialdehyde by *NAOT*, yielding N2-acetylornithine. Next, ornithine forms by

shifting the acetyl moiety to glutamate by *GAT*, imparting this enzyme a major role for continuity of ornithine cycle as it conserves the acetyl molecule (Winter et al., 2015). *GAT* is found in non-enteric bacteria (Fallik and Ilic, 2014), fungi (Miguel et al., 2004), and plants (Davis, 1986).

Subsequently, citrulline and arginine is synthesized from ornithine in a linear pathway by several coordinated enzymatic reactions namely *OCT* (ornithine carbamoyltransferase), *ASS* (argininosuccinate synthase) and *ASL* (argininosuccinate lyase) (Micallef and Shelp, 1989; Shargool et al., 1988; Winter et al., 2015). *OCT* delivers the third N-atom by carbamoylation of the δ -amino group of ornithine, forming citrulline. This reaction requires carbamoyl phosphate, which is generated from ATP, bicarbonate and the δ -amino group of glutamine by carbamoyl phosphate synthetase enzyme (*CPS*) (Winter et al., 2015). Aspartate yields fourth N-atom that is ligated to citrulline through enzyme encoded by one of the members of *ASSY* gene family. Lastly, fumarate splits off into arginine, a final product of this cycle by *As11* and *As12*. Previously, grafting was used for crop improvement in horticulture industry. Recently, it has gained tremendous importance in horticulture industry globally for minimizing the negative effects of metals, drought, flooding, disease, pests and to produce positive effects on flavor and health-related compounds (Lee, 1994). Influence on physiological processes especially secondary metabolism, photosynthesis, hormones, nutrients, water uptake and translocation of a metabolite is primarily dependent on rootstock-scion interaction (Rouphael et al., 2010). Grafting enhances plant growth thus has a positive impact on vegetable quality by up surging the quantity of health benefit compounds (Colla et al., 2010; Davis et al., 2008; Lee et al., 2010; Rouphael et al., 2008; Savvas et al., 2010). Studies have shown that grafting can affect metabolism of secondary metabolites such as lycopene, carotenoids and ascorbic acid in watermelon (Petropoulos et al., 2014; Proietti et al., 2008).

Citrulline has become a key indicator of watermelon fruit quality along with lycopene, carotenoids and other nutrients. At present, limited information is available regarding influence of grafting on citrulline content of watermelon fruits. (Soteriou et al., 2014) reported the increase in citrulline content following the grafting. In another study, grafting was reported to increase the citrulline content by 12.5% in watermelon (Kyriacou et al., 2016). Citrulline contents increase steadily with fruit development and decline in over-mature fruit (Guo et al., 2013). Previous researches mainly focused on the comparison of citrulline among different parts of watermelon fruit or its relation to cultivar, location, fruits size and ploidy levels (Joshi and Fernie, 2017). Previous studies only measured the citrulline content but underlying molecular mechanism is unknown in grafted watermelon.

According to our knowledge, this is the first report deciphering the role of grafting in regulating citrulline metabolism at transcriptional level. The mystery about the regulation of citrulline biosynthetic pathway genes is unknown in grafted watermelon. Recently drafted

watermelon genome provides a great opportunity for in-depth studies. This study aims to investigate the impact of various rootstocks on citrulline accumulation in developing fruit and transcriptional regulation of key genes of the citrulline biosynthetic pathway. It is important to understand the mechanism by which rootstock regulate citrulline content, in order to provide a reference for the production of high citrulline fruits and this knowledge can be utilized in the breeding of high citrulline producing rootstock. It will also open up the ways in search of a candidate gene for citrulline.

2. Material and Methods

The experiment was performed under a plastic greenhouse from March to July (2018) at Horticultural Research Station in Xixiang County, Henan, China. Watermelon [*C. lanatus* (Thunb.) Matsum. & Nakai var. *lanatus*] local diploid landrace scion “Zhongyu No. 1” a commercial yellow flesh mini watermelon was grafted onto the three rootstock pumpkin “Xi jia qiang sheng” (*Cucurbita moschata*), wild watermelon “Yong shi” (*C. amarus*) and bottle gourd “Chao Feng F1” (*Lagenaria siceraria*). When pumpkin was used as rootstock it was abbreviated as PGW (pumpkin grafted watermelon), similarly WGW (wild grafted watermelon) and GGW (gourd grafted watermelon) abbreviation were used for wild and gourd rootstocks respectively. Watermelon that was not grafted served as control and abbreviated as SRW (self-rooted watermelon). All the plant materials were provided by the laboratory of Polyploidy Watermelon Breeding, Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences.

Seeds were sown in plastic trays containing peat moss. Top insertion method was used for grafting (Lee, 1994). Plants were transferred to the plastic greenhouse after 15 days of grafting. Treatments were replicated thrice and a randomized complete block design was used. Each block comprised of 40 plants in a single row. Plants were grown with a row to row spacing of 150 cm and plant to plant distance was kept 50 cm. Plants were trained vertically by clipping off side branches and supported with rope. Only one fruit was kept at each plant.

Commercial production practices such as fertilization, irrigation, diseases, and pest control were carried out throughout the cropping season. When the plants entered florescence, the female flowers of grafted and self-rooted were manually self-pollinated on the same day and tagged to record the number of days after pollination (DAP). Fruits were harvested at immature white (10 DAP), white-yellow flesh (17 DAP), yellow flesh (24 DAP), near to ripe (31 DAP) ripe (38 DAP) and overripe (45 DAP) stages.

Three uniform watermelon fruits from three independent plants at each developmental stage in each treatment were harvested (see Figure 1). Harvested fruits were cut longitudinally into two halves. Fruit flesh samples were collected from the center of watermelon then immediately frozen in liquid nitrogen and stored at -80°C until use.

2.1. Citrulline extraction and analysis

Frozen watermelon fruit tissues (3g/sample) were thawed and pureed using an electric glass homogenizer (DY89-2, Ningbo Science Biotechnology Co., Ltd, China) to break cell walls then samples were diluted with 4.5 ml of solution prepared by mixing pure methanol and 6M hydrochloric acid (v/v=9:1). Diluted samples were placed in a water

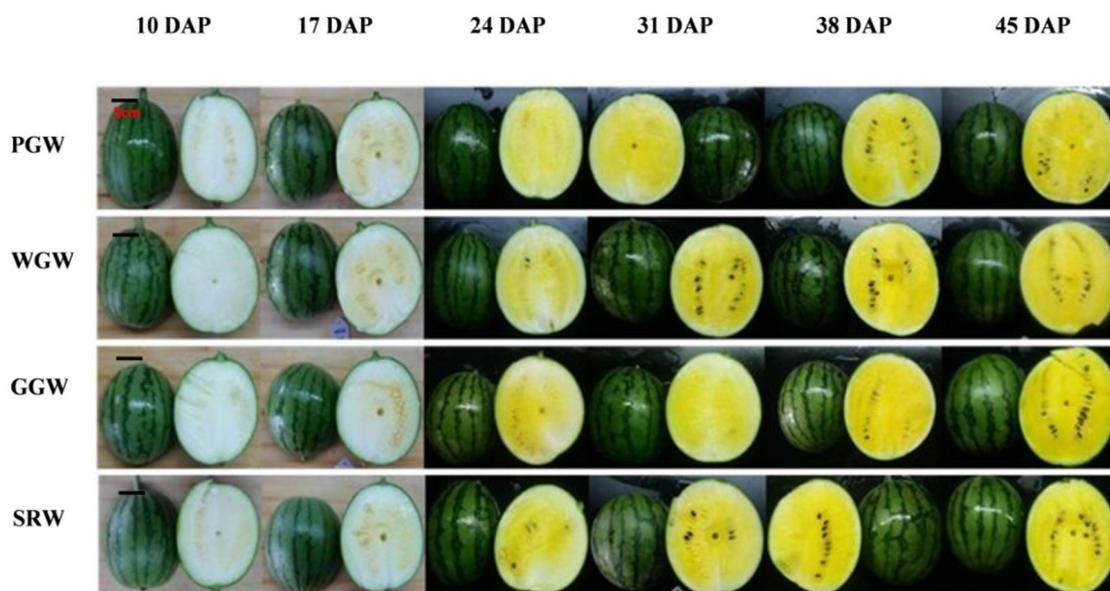


Figure 1. The fruits of pumpkin grafted watermelon (PGW), wild grafted watermelon (WGW), gourd grafted watermelon (GGW), and self-rooted watermelon (SRW) at six developmental stages, including immature white (10 DAP), white-yellow flesh (17 DAP), yellow flesh (24 DAP), near to ripe (31 DAP) ripe (38 DAP) and overripe (45 DAP).

bath for 20 min at 55°C, and then the diluted solution was decolorized by adding 1g of activated carbon and sample was filtered using Whatman #1 filter. One (1) ml of filtrate was taken and diluted with 7 ml of distilled water. Four (4) ml of the diluted solution was collected in a separate test tube, followed by addition of 2 ml of a solution prepared by mixing 3: 1 volume ratio of sulfuric acid and phosphoric acid. Afterwards, 0.25 ml (w/v= 30g/L ddH₂O) of diacetyl monoxime was added to above mixture. The solution was then heated in a 100°C water bath for 30 min in dark and allowed to cool at room temperature.

The final sample was measured with UV spectrophotometer (Beijing Lab Tech Inc. China) at 490 nm. The citrulline content was calculated according to a calibration curve of external standard citrulline (see Supplementary Material Figure 1). The data was the mean of three repeats. Citrulline content was estimated using the equation: (gkg⁻¹ · FW) = (0.096X + 0.003) × F, where A = light absorption value on 490 nm, 0.096 = slope of specification curve, 0.003 = intercept of specification curve and F = dilution rate (Cheng et al., 2010).

$$\text{Citrulline content (g / kg · FW)} = (0.096X + 0.003) \times 20 \quad (1)$$

In the equation 1, X is absorbance at 490 nm; 0.096 is the slope of the standard curve; 0.003 is the intercept; and 20 is the dilution factor.

2.2. Primer design and synthesis

Previously reported genes of citrulline pathway were selected from published literature, gene sequences were retrieved from cucurbit genomics database (cucurbitgenomics.org) by blasting protein sequence of already reported genes of the pathway. Details of gene names, species, and accession numbers are provided (see Supplementary Material Table 1). Primers for PCR amplification and real-time fluorescence quantitative PCR were generated by using ‘Primer Premier 5.0’. ‘Primer-BLAST’ was used to confirm primer specificity according to (Ye et al., 2012) by aligning the sequence of primer pairs against watermelon predicted genes (ICUGI, 2007). The reference gene used in the experiment was “Clathrin Adaptor Complex Subunit” (CLCAC) (Kong et al., 2015). The key enzyme gene names,

gene ID and primer sequences of watermelon citrulline synthetic pathway are provided (see Table 1). International Cucumber Genomic Initiative (ICUGI, 2007)

2.3. RNA extraction and gene expression analysis

Plant Total RNA Purification KIT (Gene Mark) was used to extract Total RNA from flesh tissues following the manufacturer’s instructions. RNA purity and quantity were examined using NanoDrop 2000 spectrophotometer (Thermo, China). Reverse transcription reactions were performed on 1 µg of total RNA for each sample, using Prime Script RT Reagent Kit with genomic DNA Eraser (TaKaRa, China). Light Cycler 480 System (Roche, Switzerland) was used to quantify genes expressions. Each 20 µL qRT-PCR reaction mixture containing 1 µL cDNA, 1 µL forward primer (10 µmol L⁻¹), 1 µL reverse primer (10 µmol L⁻¹), 10 µL 2× SYBR Green real-time PCR mix, nuclease-free water to final volume of 20 µL, was preheated at 95°C for 5 min, followed by 45 cycles of 95°C (10s), 55-64°C (10s) and 72°C (20s). A melting curve was generated after amplification to determine its specificity. Three biological replicates were run for each reaction.

2.4. Statistical analysis

The raw data of qRT-PCR were analyzed by using LCS480 software 1.5.0.39 (Roche, Swiss) and relative expressions of the genes were determined according to the 2^{-ΔΔCT} method (Livak and Schmittgen, 2001). Each result shown in figures or tables was the mean value from three biological repeats. The significant differences between treatments were statistically evaluated by the standard error. Analysis of variance and Pearson correlation coefficient was performed and tested for statistical significance using Statistics 8.1. Least Significant Difference (LSD) test was used for the comparison among treatment’s means at P < 0.05.

Citrulline accumulation in grafted and self-rooted watermelon

Clear differences in citrulline contents were noticed during the course of fruit development among the grafted and self-rooted watermelons (see Figure 2). Citrulline accumulation pattern was similar in all treatments. First, citrulline content increased up to 24 DAP, afterwards decreased, and finally reached the highest level at 45 DAP.

Table 1. Description of citrulline pathway key genes and primers of real-time fluorescence quantitative RT-PCR.

Gene	Gene ID	Forward Primer (5'-3')	Reverse Primer (5'-3')
NAGS	Cla014036	GTGAGCGGAATAGCCATAT	CTCCTCCAATAGTGCCATC
NAGK	Cla022273	CTCAATCCATCCGCATCC	AATCAACTACAGCCTCCATT
GAT	Cla017879	TTCACCGCCTTTCAATCC	CTCCATCAATCTGCTTCC
OTC	Cla020781	CCTGACGGATTACAACCAT	TGATGCCAACAACAACCA
ASS2	Cla002609	GCTCTACAAGGGTTCGGTCC	AGGCCGTAAAGGCGAATGAA
ASS3	Cla019267	AGATAACCGCAACAACCA	GCCGTAGAGATGAATGAATC
ASL1	Cla023055	TGCCAGAGTCATAGGAGA	CTTCTTCACGAGGTAATCAG
ASL2	Cla022154	TTGCGATGGTAGACTTGG	TGCTAAGAGAAGATGCTGAA
ARG	Cla006970	GCATCTCTAACACTATTTCG	GGACTACCAACAATCATG
CLCAC	Cla016178	GAAC TTGGCACCTGTCTTGT	GAACAGTGCAACAGCCTCAA
NAGPR	Cla010271	TACGGAATTACCCGCCATCG	TCCTCGGTAGTCATCCCTGG
NAOAT	Cla015337	GTACGATCTCGAAGGGCGAG	ACGTGAGAGACGGTATTTCGC

At these two time points, highest citrulline was observed in SRW followed by WGW and PGW. However, GGW synthesized the lowest citrulline at these two time points. At the immature stage (10 DAP) highest citrulline was recorded in the fruits of PGW followed by WGW and GGW which were significantly higher than self-rooted watermelon. Similarly, at the 17 DAP fruits of PGW accumulated a significantly higher level of citrulline as compared to other treatments, whereas amount of citrulline content was same in both WGW and GGW at 17 DAP which was also significantly higher than SRW.

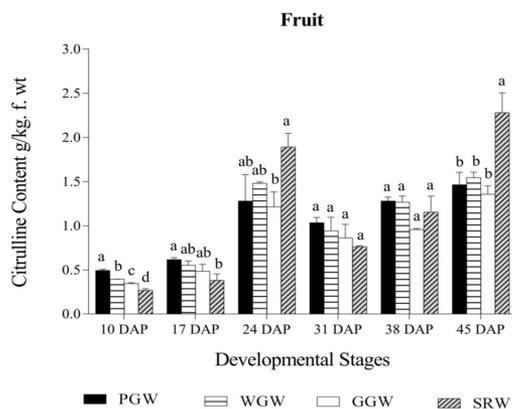


Figure 2. Citrulline contents in the fruits of grafted and self-rooted watermelon. Data are the mean + SE of three replicates. Different lowercase letters indicate significant differences at $P < 0.05$.

At the yellow flesh stage of 31 DAP no significant differences were observed on citrulline contents in the fruits of grafted and self-rooted watermelon but relative citrulline contents were higher in grafted watermelon at this time point.

At the ripe stage of 38 DAP, the same amount of citrulline content was detected in the fruits of PGW and WGW, which was higher than that of self-rooted. However, the GGW fruit synthesized the lowest citrulline content at this stage.

Overall, among the four treatments, citrulline contents were highest at all stages in PGW with an exception of 24 and 45 DAP. Whereas citrulline content was only highest in SRW during the middle of fruit development (24 DAP) and in overripe fruit (45 DAP).

Lowest citrulline content was observed in GGW at 24, 38 and 45 DAP. Grafting increased the citrulline content especially when pumpkin and wild rootstocks were used.

2.5. Expression analysis of citrulline biosynthesis genes

We assessed the relative expression levels of key genes encoding enzymes of citrulline pathway at six developmental stages and correlated it with citrulline content to understand regulation of pathway over time in various rootstocks (see Table 2 and Figure 3). All of genes expressed at all the stages in watermelon scion grafted onto various rootstocks.

Genes involved in biosynthesis of citrulline showed various trends in grafted and self-rooted watermelon. Grafting suppressed the expression of all the biosynthesis genes and suppression was more pronounced in WGW and GGW. In PGW, the expressions for *NAGS*, *NAGK*, *NAOT*,

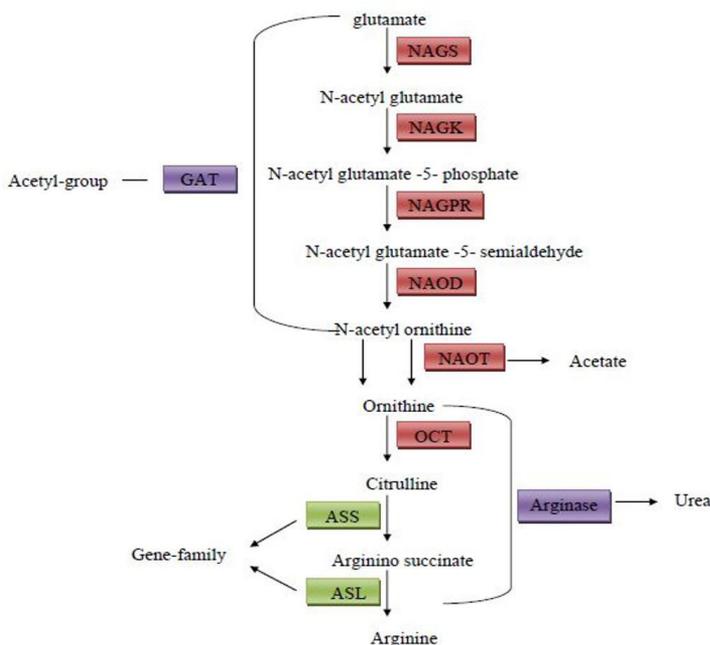


Figure 3. Modified citrulline biosynthesis pathway (Slocum, 2005; Winter et al. 2015; Takahara et al., 2005). *NAGS*-N-acetylglutamate synthase; *NAGK*-N-acetylglutamate kinase; *NAGPR*-acetylglutamate 5-phosphate reductase; *NAOAT*-acetylornithine transaminase; *GAT*-glutamate N acetyl transferase; *NAOD*-acetyl ornithine deacetylase; *OCT*-ornithine carbamoyl transferase; *ASS*- argininosuccinate synthase and *ASL*-argininosuccinate lyase.

OCT, *GAT*, and *NAGPR* were substantially lower and exhibited a decreasing tendency towards the subsequent fruit development stages as compared to self-rooted watermelon. Noticeably, in self-rooted watermelon transcript abundance of all biosynthesis genes were significantly higher than all other treatments and expressions of biosynthesis genes were inconsistent throughout the development of fruit. Additionally, *GAT* showed highly significant correlation

with citrulline content in PGW whereas, in SRW no gene was found to be correlated with citrulline content. Interestingly, in WGW and GGW all the biosynthesis genes of the pathway expressed at an extremely low level at each stage as compared to SRW and PGW, showed relatively stable expressions and the trends were similar for all the biosynthesis genes (see Figure 4). Expressions of *NAGS* and *GAT* were highly correlated with citrulline

Table 2. Correlation analysis of citrulline metabolic genes with citrulline content during the development of watermelon fruit.

Gene Name	Correlation Coefficients			
	PGW	WGW	GGW	SRW
<i>NAGS</i>	-0.139 ^{NS}	-0.519*	-0.000 ^{NS}	-0.137 ^{NS}
<i>NAGK</i>	-0.169 ^{NS}	-0.358 ^{NS}	-0.571*	0.166 ^{NS}
<i>NAOT</i>	0.028 ^{NS}	0.160 ^{NS}	0.296 ^{NS}	0.130 ^{NS}
<i>GAT</i>	-0.690***	-0.497*	-0.683***	-0.112 ^{NS}
<i>OTC</i>	-0.052 ^{NS}	-0.281 ^{NS}	-0.400 ^{NS}	-0.358 ^{NS}
<i>ASS2</i>	-0.533*	-0.425 ^{NS}	-0.540*	-0.295 ^{NS}
<i>ASS3</i>	-0.488*	-0.360 ^{NS}	-0.618**	-0.298 ^{NS}
<i>ASL1</i>	0.037 ^{NS}	-0.361 ^{NS}	-0.638**	-0.473*
<i>ASL2</i>	-0.631**	-0.775***	-0.340 ^{NS}	-0.545*
<i>ARG</i>	-0.068 ^{NS}	-0.246 ^{NS}	-0.501*	-0.448 ^{NS}
<i>NAGPR</i>	-0.770***	0.290 ^{NS}	0.150 ^{NS}	-0.229 ^{NS}
<i>NAGS+NAGK+NAOT+NAGPR+GAT+OTC</i>	-0.182 ^{NS}	-0.417 ^{NS}	-0.319 ^{NS}	-0.176 ^{NS}
<i>ASS2+ASS3+ASL1+ASL2+ARG</i>	-0.676**	-0.3814 ^{NS}	-0.4123 ^{NS}	-0.4230 ^{NS}
<i>ASS2+ASS3+ASL1+ASL2</i>	-0.680***	-0.3794 ^{NS}	-0.4090 ^{NS}	-0.3939 ^{NS}

asterisk indicates significance level where; * represents $p < 0.05$; ** represents $p < 0.01$ and *** represents $p < 0.001$.

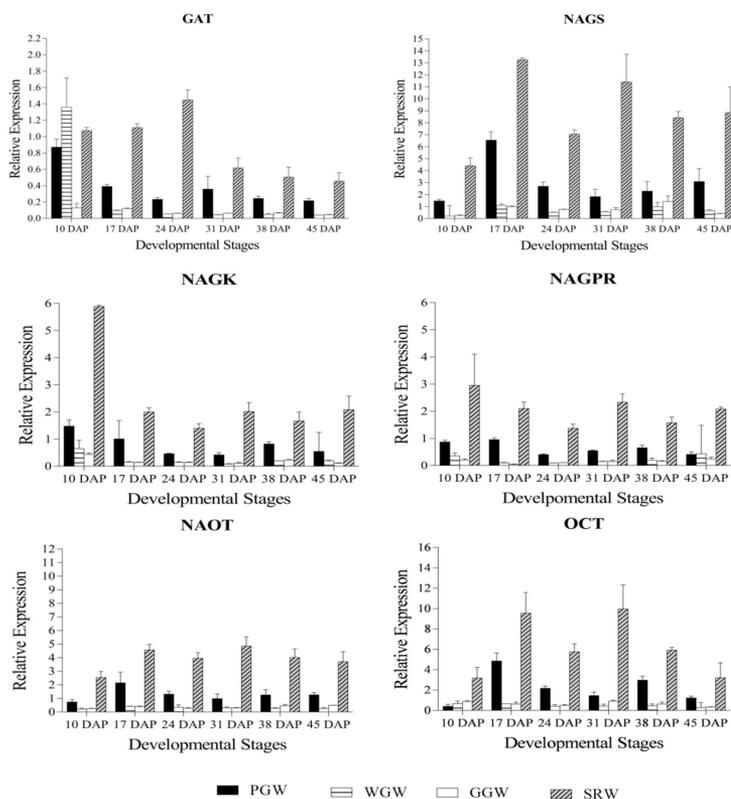


Figure 4. Relative expression of citrulline biosynthesis genes involved in citrulline pathway during fruit development and ripening of the grafted and self-rooted watermelon. Each value is presented as the means + standard error (S.E.), n=3.

content in WGW while in GGW *GAT* and *NAGK* showed good correlation with citrulline content. To evaluate the additive effect of the all biosynthesis genes in the citrulline pathway, expression profiles of the *NAGS*, *NAGK*, *NAGPR*, *NAOT*, *OCT* and *GAT* was added for each rootstock separately and correlation analysis was performed with citrulline content. Interestingly, no strong correlation was found, indicating genes work independently. These results suggested that rootstocks used different strategies to regulate the citrulline content.

2.7. Expression analysis of citrulline catabolic genes

Most of the catabolic genes were relatively down regulated with the fruit development in grafted and self-rooted watermelon but transcript abundance was different for various treatments used (see Figure 5). The similar expression profiles for *ASS2* and *ASL2* were observed in all the treatments and showed high down regulation with progress in development of fruit.

Expressions of *ASS2* and *ASL2* were highest in PGW only at an immature stage of 10 DAP as compared to all other treatments. From 17 DAP to 45 DAP expressions of *ASS2* and *ASL2* were higher in self-rooted while the lowest expressions were recorded for WGW and GGW. Correlation analysis predicted strong relation of *ASS2* and *ASL2* with citrulline in PGW. While *ASL2* and *ASS2* were significantly correlated with citrulline in WGW and GGW respectively.

Expressions for *ASS3* and *ASL1* were higher in self-rooted watermelon followed by PGW during the development stages lagging behind other treatments. In the self-rooted watermelon *Ass3* and *Asl1* were first down-regulated up to 24 DAP and then showed little increase in later stages of fruit development while expressions for *ASS3* and *ASL1* were relatively low and consistently down-regulated throughout the developmental in PGW as compared to self-rooted watermelon. Expression patterns of *ASS3* and *ASL1* were similar in the fruit of WGW and GGW and substantially down regulated with development of fruit. However,

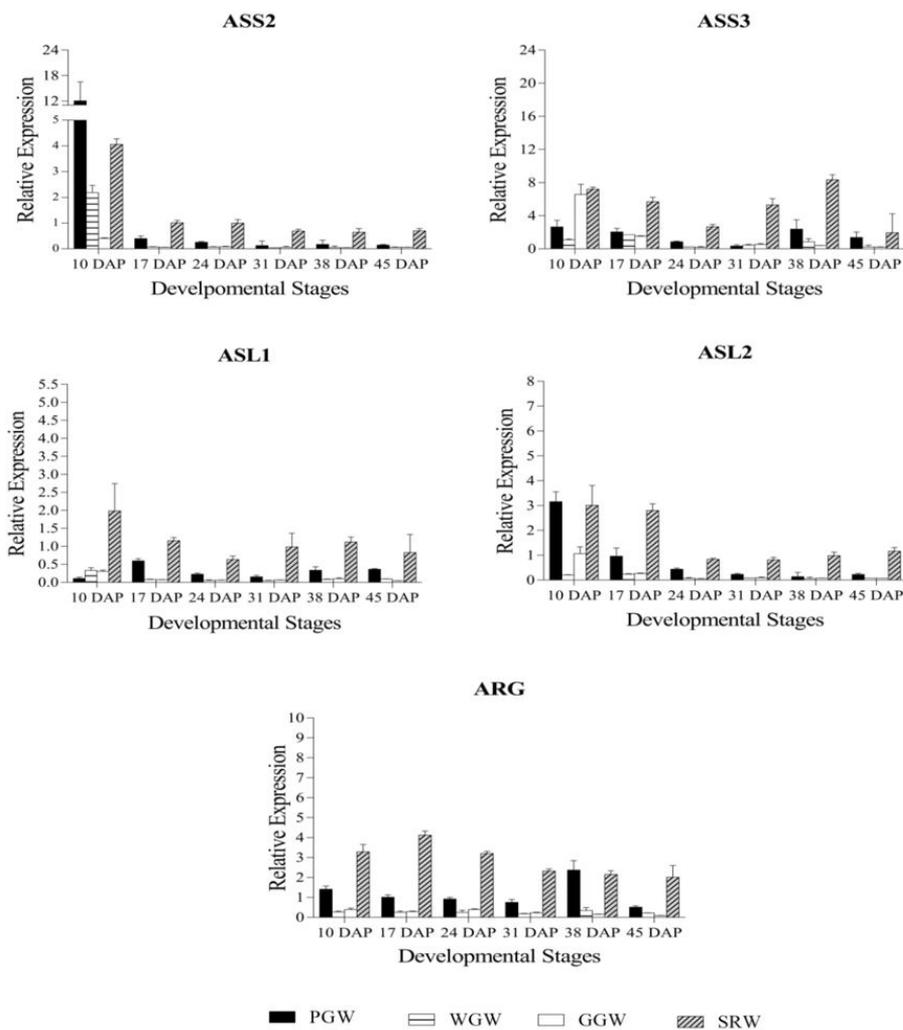


Figure 5. Relative expression of citrulline catabolic genes involved in citrulline pathway during fruit development and ripening of the grafted and self-rooted watermelon. Each value is presented as the means + standard error (S.E.), n=3.

ASS3 and *ASL1* expressed at extremely low level in the fruit of WGW and GGW as compared to PGW and SRW. A catabolic gene *ASS3* was found to be highly correlated with citrulline in PWG while two genes *Ass3* and *Asl1* showed high correlation with citrulline in GGW.

Finally, the *ARG* was down-regulated up to 31 DAP in PGW then peaked at 38 DAP where its expression was highest than other treatments. WGW and GGW showed similar expression profiles for *ARG* and expression was stable along the developmental stages while it was down-regulated in self-rooted watermelon and expression was higher at all stages except at 38 DAP as compared to other treatments (see Figure 5).

Correlation analysis was carried out on additive expression of all the catabolic genes to elucidate their additive role in each rootstock independently. Similarly, expression profiles of two gene families *ASS2*, *ASS3*, *ASL1* and *ASL2* were added up and correlation analysis was performed with citrulline contents. Results demonstrated that in PGW, additive expression of all catabolic genes was significantly correlated with citrulline. Similarly, additive gene expression of two gene families *ASS* and *ASL* was also highly correlated with citrulline suggesting synergistic effect of this gene family. Results showed that different rootstocks used different mechanisms to regulate the genes expressions which in turn influence the citrulline contents.

3. Discussion

Grafting has been in practice since a long time to combat against soil-borne pathogens, diseases and to improve the fruit quality, yield and nutrient uptake (Condurso et al., 2012; Fallik and Ilic, 2014; Miguel et al., 2004; Roupael et al., 2010). Grafting as a tool to manipulate the fruit quality and yield has gathered importance recently (Condurso et al., 2012; Davis and Perkins-Veazie, 2005; Davis et al., 2008). Grafting has impact on citrulline content but underlying molecular mechanism has never been explored in grafted watermelon.

In this study, we observed that citrulline steadily increase with development up to 24 DAP then decreased but it again increased in over-mature fruit which is in partial agreement with results of (Guo et al., 2013) who reported that citrulline content progressively increases up to 26 days post-anthesis and thereafter decreased suddenly. However, progressive increase in citrulline content from 30 to 45 DAP has also been reported by (Soteriou et al., 2014). Moreover, minimum citrulline content was recorded in immature fruit of 10 DAP, a similar trend was observed for lower citrulline content at early or immature fruit stage but it increased towards the fruit maturity steadily and decreased with ripening (Breuillard et al., 2015; Fish, 2014; King, 2009). The differences in reported results may be due to different type of rootstocks, interactions between specific rootstocks and scions, and harvesting date (Davis et al., 2008).

Citrulline content progressively increased from mature to overripe stage in the fruit of PGW, WGW and GGW.

Citrulline is regulated with the development of fruit, citrulline content increased with ripening and reached the highest level 45 days after post anthesis in grafted watermelon (Soteriou et al., 2014). Similar findings were reported by (Kyriacou et al., 2016) that grafting increased the citrulline content by 12.5% and reached a climax near the fruit maturity. The results mentioned above indicated that different root-scion stocks combination communicated differently to regulate the citrulline content.

Our results demonstrated that rootstock induced changes in citrulline content is not in complete parallel with expression of key genes in citrulline pathway suggesting that the pathway is partially regulated at the transcriptional level. However, rootstocks significantly suppressed the expression of most of the genes studied and suppression was more pronounced in WGW followed by GGW, suggesting that pathway is not only regulated at the transcriptional level some other regulatory mechanism could be involved. For instance, *NAGS* and *NAGK* are considered as a regulatory enzyme for ornithine production (Slocum, 2005). Both are the target of feedback inhibition by downstream citrulline or arginine (Kalamaki et al., 2009). Plastidic PII which is signal transduction protein-mediated feedback regulation of *NAGK* and *NAGS* (Bourrellier et al., 2009; Burillo et al., 2004; Heinrich et al., 2004; Ll acer et al., 2008; Slocum, 2005).

In Arabidopsis, arginine production (a downstream product of citrulline) has been suggested to be regulated by high nitrogen availability, which in turns regulated the catalytic properties of *NAGK*, PII protein complex and its sensitivity towards arginine (Cheng et al., 2010; Heinrich et al., 2004; Ll acer et al., 2008; Maheswaran et al., 2004; Slocum, 2005). Differential gene expression for *NAGS* and *NAGK* among various treatments used may be explained by the fact that rootstock have been reported to absorb differential amount of N<P<K supply which in turn has affected the *NAGK* and has increased the feedback inhibition and thus reducing the expression in grafted watermelon and it is an accepted fact that different rootstock perform differently as elaborated in previous findings where rootstock enhanced capacity for N uptake and nitrogen use efficiency in watermelon (Colla et al., 2011; Pulgar et al., 2000). Nevertheless, expression of *NAGS* and *NAGK* were highly correlated with citrulline accumulation in WGW and GGW respectively.

Down regulation of *NAOGAcT* (Cla017879) was responsible for high accumulation of citrulline in the leaves of *C. colocynthis* under drought stress (Wang et al., 2014). In this study, *GAT* was down regulated and showed negative correlation with citrulline in all grafted watermelon. On contrary to these results, (Takahara et al., 2005) reported the up-regulation of *GAT* was associated with high citrulline content under drought and high-temperature stress and proved its insensitivity to feedback inhibition caused by citrulline. A similar pattern was observed for *NAGPR* in grafted watermelon. *NAOT* and *NAGPR* genes expressed at all the stages and expressions were higher in self-rooted as compared to grafted. However, expression of *NAGPR*

was highly correlated with citrulline in PGW. High level of ornithine and arginine may have influenced enzyme activity or gene expression by feedback mechanism as for *NAGS* and *NAGK* (Slocum, 2005) *OCT* expression decreased and down-regulated at most of the stages in grafted watermelon. Similar results were reported by (Guo et al., 2013). Lower expression of *OCT* correlated with high citrulline content. Previous studies have suggested that *NAGS*, *NAGK*, *OCT*, *NAOT* and *NAGPR* genes play a key role in citrulline pathway (Takahara et al., 2005). Oppose to this, in our study additive expressions of these genes was not significantly correlated with citrulline content in all treatments, difference in results may be attributed to different treatments under consideration. *ASS2* and *ASS3* were highly down-regulated in all treatments and expression level decreased markedly in pumpkin, wild and gourd grafted watermelons during the development which aligns with high citrulline content at an early stage, the stage before the ripening and at ripe stage.

The down-regulation of genes encoding *ASS2* and *ASS3* in watermelon is consistent with previous findings (Guo et al., 2013). The abrupt increase in citrulline in grafted and self-rooted watermelon at 24 DAP and 45 DAP aligns with a parallel decrease in expression of *Ass3* and *Ass2*, especially in self-rooted watermelon. Our results demonstrated that this gene family works synergistically which probably led to decrease degradation of citrulline which is also reported by (Guo et al., 2013). Among all the genes studied *As11* and *As12* (gene family) were also down-regulated in all treatments and transcript abundance was lowest in grafted watermelon which is in consensus with (Guo et al., 2013). Additionally, studies have also shown that the *ASL* gene mutation reduced the arginine content in rice (Frémont et al., 2013). These findings suggested that *As11* and *As12* also worked synergistically resulting in high accumulation of citrulline. In this study correlation analysis also predicted synergistic effect of two genes families *ASS* and *ASL* and that aligns with higher citrulline content in PGW. *As13* showed significant correlation with citrulline in WGW whereas, *ASS2*, *ASS3*, *ASL1* and *ARG* were strongly correlated with changes in citrulline content in GGW. Arginase expression was low and down-regulated in all grafted watermelon but in GGW fruits *ARG* showed strong inverse correlation which is in accordance with (Guo et al., 2013). Over-expression of the *ARGAH2* gene in *Arabidopsis* increased the arginine and ornithine contents (Brauc et al., 2012).

4. Conclusion

Citrulline seems to be a rootstock dependent trait. Rootstock played a key role in mediating the citrulline metabolism through transcriptional regulation in different ways. Increase in citrulline content during the development of pumpkin grafted fruit was associated with high down regulation of two gene families *ASS* and *ASL* which resulted in decreased breakdown of citrulline. In wild grafted watermelon, citrulline accumulation was attributed

to down regulation of *GAT*, *NAGS* and *ASL2*. In gourd grafted watermelon, *GAT*, *NAGK*, *ASS2*, *ASS3*, *ASL1* and *ARG* were substantially down-regulated which resulted in higher citrulline content. However, lower citrulline content in self-rooted watermelon attributed to higher expressions of several biosynthesis gene and down regulation of *ASL1* and *ASL2*. This study provides an alternative approach to improve the citrulline content through use of various rootstocks.

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Supplementary Material

Supplementary material accompanies this paper.

Figure 1. The standard calibration curve of citrulline with spectrophotometric method.

Table 1. Previously reported genes of citrulline in other crops.

This material is available as part of the online article from <http://www.scielo.br/BJB>