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EXPRESSION ANALYSIS OF GENES ENCODING RHAMNOGALACTURONAN LYASE ISOENZYMES DURING TOMATO FRUIT DEVELOPMENT AND RIPENING

ANÁLISIS DE EXPRESIÓN DE GENES CODIFICANTES DE ISOENZIMAS DE RAMNOGALACTURONANO LIASA DURANTE EL DESARROLLO Y LA MADUREZ DEL FRUTO DE TOMATE

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SUMMARY

Tomato cultivation generates great profits for Mexico; however, the distribution of the fruit faces limitations due to the postharvest losses caused by its rapid deterioration. The reduction in fruit firmness is due in part to the degradation of pectin located in the primary cell wall. Several fruits such as strawberry (*Fragaria × ananassa*), tomato (*Solanum lycopersicum* L.) and mango (*Mangifera indica* L.) show an increase in the expression of genes that code for the enzyme rhamnogalacturonan lyase (RGL) during ripening. In tomato RGL is encoded by a family of 13 multigenes, of which three are active in the fruit. Rhamnogalacturonan I (RG-I) is a polysaccharide that is part of the pectins of the cell wall and is degraded by the RGL enzyme. Thus, the study of changes of RGL in the expression of genes that code for RGL during the development and ripening of tomato fruit will help to elucidate the physiological function of this family of genes. The objective of the present study was to assess the expression profile of genes *Solyc04g076630, Solyc04g076660* and *Solyc11g011300* and the ethylene production during the development and ripening of the tomato fruit. The *Solyc11g011300* gene showed an increase in activity that correlated with the increase in ethylene production in fruits, suggesting that this gene plays a role in the loss of firmness of the fruit during ripening. The high levels of expression of genes *Solyc04g076630* and *Solyc04g076660* recorded during fruit development suggest their participation in the re-engineering of the RG-I polymer during the initial stages of fruit development.

Index words: *Solanum lycopersicum*, cell wall, ethylene, pectin, rhamnogalacturonan-I, rhamnogalacturonan lyase.

RESUMEN

El cultivo de tomate genera grandes ganancias para México; sin embargo, la distribución del fruto enfrenta limitaciones debido a pérdidas postcosecha causadas por su rápido deterioro. La reducción en la firmeza del fruto se debe en parte a la degradación de la pectina localizada en la pared celular primaria. Varios frutos como fresa (*Fragaria × ananassa*), tomate (*Solanum lycopersicum* L.) y mango (*Mangifera indica* L.) muestran incremento en la expresión de genes que codifican para la enzima ramnogalacturonano liasa (RGL) durante la maduración. En tomate RGL es codificada por una familia de 13 multigenes, de los cuales tres están activos en fruto. Ramnogalacturonano I (RG-I) es un polisacárido que forma parte de las pectinas de la pared celular y que es degradado por la enzima RGL. En este sentido, el estudio de los cambios en expresión de genes que codifican para RGL durante el desarrollo y maduración del fruto de tomate ayudará a dilucidar la función fisiológica de esta familia de genes. El objetivo del presente estudio fue evaluar el perfil de expresión de los genes *Solyc04g076630, Solyc04g076660* y *Solyc11g011300* y la producción de etileno durante el desarrollo y maduración del fruto de tomate. El gen *Solyc11g011300* mostró un incremento en actividad que correlacionó con el aumento en la producción de etileno del fruto, lo cual sugiere que este gen cumple una función en la pérdida de firmeza del fruto durante maduración. Los altos niveles de expression de los genes *Solyc04g076630* y *Solyc04g076660* registrados durante el desarrollo del fruto sugieren su participación en la re-ingeniería del polímero RG-I durante las etapas iniciales de desarrollo del fruto.

Palabras clave: *Solanum lycopersicum*, etileno, pared celular, pectina, ramnogalacturonano-I, ramnogalacturonano liasa.

INTRODUCTION

Mexico is the most important producer and exporter of vegetables globally, which creates high revenues for the country. For instance, Mexico exported 2.17 million tons of tomato (*Solanum lycopersicum* L.), which is equivalent to 2613 million US dollars (SAGARPA, 2017); however, approximately 25 % of this production is lost due to several factors, including physical injuries, overripening, fungal infection and physiological disorders (Kitinoja *et al*., 2018). Fresh fruit quality is mainly based on organoleptic and quality properties, with firmness being a determinant quality for customer acceptance. In this context, in the last decade, several scientific studies have focused on delaying fruit softening by studying the cell wall (Rongkaumpan *et al*., 2019), its composition and structure (Segado *et al*., 2016), and the genes (Belouah *et al*., 2020) and enzymes involved in cell wall polymer degradation (Uluisik and Seymour, 2020). In many of these publications, tomato was used as a model because it has a relatively short life cycle and produces a fleshy fruit. Additionally, the tomato genome is known and characterized, and there are several

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tools available to study its biology.

The plant cell wall (PCW) comprises three domains: cellulose, hemicellulose and pectin. Pectin is the most complex and dynamic domain, it is composed of the polysaccharides homogalacturonan, rhamnogalacturonan I (RG-I) and rhamnogalacturonan II. RG-I is a polysaccharide whose backbone is constituted by repeated moieties of rhamnose and galacturonic acid joined by a glycosidic bond with the conformation a-L-Rhap-(1,4)-a-D-GalpA (McDonough *et al*., 2004). RG-I is degraded by the rhamnogalacturonan lyase (RGL) enzyme through a β-elimination mechanism. Although the biochemical mechanism of this enzyme is well known, the physiological role of RGL during fruit ontogeny is not yet fully understood.

The activity of the RGL enzyme has been reported to be involved in cell wall enlargement by changing the cohesion network in cotton cotyledons (*Gossypium hirsutum* L.) (Naran *et al*., 2007), the activation of the defense system in tomato (Jiménez-Maldonado *et al*., 2018), the regulation of cell division and periderm development (Oomen *et al*., 2002) and the control of cell wall architecture in potato (*Solanum tuberosum)* (Huang *et al*., 2017). Recently, it was reported that RGL isoenzymes are involved in the deposition of tertiary cell wall fibers in flax (*Linum usitatissimum*) (Mokshina *et al*., 2019). Furthermore, there is evidence to suggest the activation of RGL isoenzymes during fruit ripening in strawberry (Méndez-Yañez *et al.,* 2020; Molina-Hidalgo *et al.,* 2013) and mango (Dautt-Castro *et al.,* 2015; Tafolla-Arellano *et al.,* 2017), this suggests the possible activation of some of the RGL isoenzymes in rippening. In addition, bioinformatics analysis of the different response elements in the promoter region of 13 genes encoding RGL in the tomato genome was carried out. This analysis suggested that RGL might play a role in cell expansion, plant growth and development, fruit ripening, fruit softening, and pollen tube development (Berumen-Varela *et al.* (2017).

A previous study reported that the gene *Solyc11g011300* was expressed during fruit ripening by using a construct in which the β-glucuronidase gene was under the control of the *Solyc11g011300* promoter (Berumen-Varela *et al.,* 2018). Furthermore, the overexpression of the *Solyc11g011300* gene in tomato *S. lycopersicum* cv. Ohio 8245 suggested that this gene plays a role in the change in firmness of tomato fruits (Ochoa-Jiménez *et al.,* 2018). Additionally, analysis of the promoters of the *Solyc04g076630*, *Solyc04g076660* and *Solyc11g011300* genes showed the presence of the ethylene response elements ERELE4 and ERF1 (Berumen-Varela *et al.,* 2017); however, it is necessary to assess changes in the expression levels of these genes in wild-type fruit during development and ripening to determine whether ethylene could be associated with the expression of these genes and to propose the physiological function of these three genes in the remodeling or dismantling of the cell wall, which constitute the objectives of the present study.

MATERIALS AND METHODS

Plant material

Tomato plants (*S. lycopersicum*) cv. Rutgers were grown under greenhouse conditions at Zamora, Hermosillo, Sonora, México (20° 16′ 56.8″ N, 110° 53′ 08.5″ W). Inside the greenhouse, temperatures ranged from 25 to 32 °C. Tomato plants were watered daily, with weekly applications of a Hoagland solution enriched with 300 mM H_3PO_4 and 15 mM K_2SO_4. Self pollination was carried out by gently moving unpollinated flowers using a small brush. Immediately thereafter, a cardboard tag including the date was placed in the peduncle tissue. Tomato fruits were collected at 5, 10, 30 and 40 days after anthesis (DAA). Afterwards, tomato fruits were harvested at the mature green (MG), turning (TUR) and red ripe (RR) stages based on the United States Standards for Grades of Fresh Tomatoes (CFR51.1855-51.1877). In all samples, the fruit mesocarp was removed, frozen with liquid nitrogen and kept at -80 °C for further analysis.

Analysis of gene sequences

Amino acid sequences of the isoenzymes *Solyc04g076630*, *Solyc04g076660* and *Solyc11g011300* were obtained from the SolGenomics Network (SGN). Sequences were analyzed for the presence of domains using domain finder from NCBI (ncbi.nlm.nih.gov/Structure/cdd/wrpsb) and aligned with COBALT (Constraint-based Multiple Alignment Tool) from NCBI and Clustal-Omega from EMBL-EBI. The Expasy box shade online tool was used to represent the alignment. The reference sequence was rhamnogalacturonan lyase (GenBank id QFG75912.1).

Ethylene quantification

Ethylene was quantified based on the methodology published by Ojeda-Contreras *et al*. (2008). For the assay, three biological samples composed of three tomato fruits were analyzed. Tomatoes were incubated for 1 h at 25 °C in a 1 L sealed glass container. After that time, fruit ethylene production was measured by collecting a 1-mL sample from the headspace with a syringe and injecting it into a chromatograph (Varian 3400 cx gas, Agilent Technologies, Santa Clara, California, USA) equipped with a HayeSep N column that was 2-m long with an internal diameter of

3.17 mm (Supelco Analytical, Inc., Bellefonte, Pennsylvania, USA) equipped with a thermal conductivity detector for CO_2 quantification and a flame ionization detector for ethylene quantification. The chromatography conditions were as follows: injection temperature of 100 °C, thermal conductivity detector temperature of 170 °C, and flame ionization detector temperature of 120 °C. Nitrogen was used as the carrier gas with a flow rate of 25 mL min^{-1}.

RNA Isolation and first strand DNA synthesis

The pericarp of fruits at different DAA was isolated, chopped into small pieces, frozen in liquid nitrogen, pulverized with a frozen mortar and pestle, and then stored at -80 °C until further analysis. Three tomato fruits harvested from individual plants were considered a single biological sample, and two biological samples were used for the analyses. RNA was isolated with the hot borate method (Wilkins and Smart, 1994). RNA was quantified in an ultra-low volume spectrophotometer (Nanodrop 2000, Thermo Scientific, Waltham, Massachusetts, USA), and then RNA was treated with DNAse RQ1 (Promega) according to the manufacturer's instructions. RNA integrity was corroborated by gel electrophoresis on a 1.2 % agarose gel. First strand DNA was synthesized from 1 µg of RNA, and then the retrotranscription reaction was performed by SuperScript II (Invitrogen) following the manufacturer instructions.

Quantification of transcripts

Primers were designed with QuantPrime (Arvidsson *et al*., 2008) using the tomato cDNA database (ITAG release 2.4). Primers were tested in a dynamic range assay. In the retrotranscription reaction, a mix of specific reverse primer pools was added for reference and RGL genes at a concentration of 400 pM for each reverse-specific primer (Nolan *et al.*, 2006). Gene for ubiquitin (UBI), a catalytic subunit of protein phosphatase 2A (PP2Acs) (Løvdal and Lillo, 2009) and a TIP41-like family protein (Expósito-Rodríguez *et al.,* 2008) were used to normalize the gene expression results (Table 1).

To quantify the RGL isoenzyme and reference gene expression levels, quantitative real-time polymerase chain reaction (RT-qPCR) was performed in a total reaction volume of 20 mL. cDNA was measured by a Nanodrop 2000 after elimination of de RNA with RNAase H. Further, 20 ng of cDNA were used as a template for each reaction; then, 250 nM forward and reverse primers were used for the experimental reaction. PCR reagents and 10 mL of 2X HotStart-IT SYBR Green (Affymetrix) were added to the mix. Both biological samples were measured with three technical replicates in an Real-time PCR System (StepOne, Applied Biosystems, Waltham, Massachusetts, USA) under the following conditions: 2 min at 95 °C for denaturing and 40 cycles of 15 seconds at 95 °C and 30 seconds at 60 °C. The results were calculated with the $2^{-\Delta CT}$ methodology (Schmittgen and Livak, 2008).

Statistical analysis

Expression data were analyzed under a completely randomized design with a statistical significance of $P \leq 0.05$. One-way analysis of variance (ANOVA) was used, and when ANOVA identified statistically significant results, the Tukey-Kramer test was used to identify significant difference between the means. All statistical analyses were performed by NCSS 7. For the Pearson correlation coefficient, an R script was designed to correlate the gene expression data with ethylene production during fruit ripening.

RESULTS AND DISCUSSION

In silico analysis of amino acid sequences

The functional domains between the three RGL isozymes analyzed were compared, including a characterized rhamnogalacturonan lyase (QFG75912.1) from beach strawberry (*Fragaria chiloensis)* as a reference sequence (Méndez-Yañez *et al.,* 2020). Figure 1 shows the catalytic domain, active site, and cofactor binding domain motifs in the three tomato RGL genes and in the reference rhamnogalacturonan gene from strawberry. It was recorded a 54.79, 52.73 and 57.01 of percentage identity with the reference sequence in *Solyc11g011300*, *Solyc04g076630* and *Solyc04g07660* genes, respectively. Furthermore, approximately 54 % identity was recorded among the tomato RGL sequences. The low identity is most likely because the *Solyc04g07660* and *Solyc04g076630* genes are located on chromosome 4, and the *Solyc11g011300* gene is on chromosome 11. The catalytic domain RGL_4N (accession cl15675) is responsible for cleaving the glycosidic bond (Figure 1, underlying continuous black line) between the rhamnose and galacturonic acid residues with the configuration L-rhamnopyranosyl-(1->4)-α-D-galactopyranosyluronide by a β-elimination mechanism (McDonough, *et al.,* 2004). In this domain, the residues Lys-Tyr-Leu and His are conserved in the three aligned RGL genes (Figure 1, highlighted in black). In agreement with this result, Uluisik and Seymour (2020) reported that β-elimination requires basic residues such as Arg, Lys, or His to separate the α-proton at the C5 atom of the substrate galactose residue. The middle RGL4_M (accession cd10316) and carbohydrate binding module

(CMB, accession pfam14683) are shown with underlying gray and red lines, respectively, in Figure 1. Furthermore, these modules are present in *Solyc11g011300*, *Solyc04g076630*, *Solyc04g076660* and the reference (QFG75912.1) rhamnogalacturonan gene. Additionally, the conserved calcium binding site within the CBM domain (Uluisik and Seymour, 2020) is highlighted in light green.

The main function of RGL4_M and CMB is to interact with the spatial arrangement of the RG-I polymer ramification (Silva *et al.,* 2016). These three domains were also found within the amino acid sequence of an RGL enzyme isolated from two species of strawberry (Méndez-Yañez *et al.,* 2020; Molina-Hidalgo *et al.,* 2013). In the three tomato RGL sequences, the three domains were found to play a role in cleaving and interacting with the RG-I pectin polymer of the PCW. Furthermore, the presence of the conserved amino acid residues Lys, Tyr, Leu and His in the catalytic domain was observed. Based on the above results, *Solyc04g076660, Solyc04g076630* and *Solyc11g011300* show the presence of domains indicating that they are rhamnogalacturonan lyase enzymes, although more experimental evidence is needed to support this statement.

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Gene expression and ethylene production

With the aim of elucidating the physiological function of RGL on the PCW during fruit development and ripening, the expression patterns of three genes encoding different RGL isoenzymes were obtained. Quantification of the *Solyc04g076660* transcript (Figure 2A) showed low levels of gene accumulation from 5 to 40 DAA. Furthermore, during fruit ripening, higher quantities of *Solyc04g076660* transcripts at the MG and RR stages were observed, with no statistically significant differences ($P > 0.05$). Figure 2B shows the expression changes of the *Solyc04g076630* gene. Very low expression was recorded during fruit development, except at 10 DAA, in which there was an eightfold increase in expression relative to that at 5 DAA ($P \leq 0.05$) and a more than fourfold increase relative to that at the MG stage of fruit ripening ($P \leq 0.05$).

The accumulation of transcripts from the *Solyc04g076630* gene in fruits at 10 DAA can be associated with cell wall remodeling during fruit development. In the two phases of fruit growth (fruit division and fruit expansion), the pectin polymer RG-I and other polymers constantly changed, as reviewed by Wang *et al*. (2018). This study showed increase in the *Solyc04g076630* transcript level during fruit development when cell division ended at 10 DAA (Figure 2 B). As reported by Goulao and Oliveira (2008), modification of the plant cell wall in the early stages of fruit development can be carried out by the coordinated activity of different isozyme families, as observed in the wild tomato Ailsa Craig, pear and apple. Furthermore, evidence reported by Catalá *et al.* (2000) and Wu *et al.* (1996) suggests that auxin induces the expression of enzymes that modify cell wall polymers, such as xyloglucan endo-transglycosidase (*Le*EXT1), expansin (*Le*Exp2) and endo 1-4-β-glucanase during the early stage of fruit development; at this stage, the tomato fruit diameter is between 0.5 and 3 cm and the fruit is growing mainly by cell division. In fact, the production of auxins by fruits in the cell division phase (between 5 and 10 DAA) has been reported (Mapelli *et al*., 1978). Additionally, the *Sl*ARF gene family, whose activation is regulated by auxins, contains the regulatory box TGTCTC in the promoter region, which has been shown to induce the expression of the gene *Sl*ARF5 (Liu *et al*., 2018). In this study, it is suggested that the expression of *Solyc04g076630* at 10 DAA may be due to the presence of the regulatory box TGTCTC contained in the RGL promoter (Berumen-Varela *et al.* (2017). Considering that the transcriptomic analyses showed that the *Solyc04g076630* and *Solyc04g076660* genes are expressed in the pericarp

Table 1. Reverse-specific sequences for RGL isoenzymes and reference genes used in this study.

SolGenomics ID	Primer assignation	Forward sequence 5'-3'	Reverse sequence 5'-3'
Solyc04g076630	rgl630	TACATAGTTCTTCGTGATTCGCCT	CGCGAAACTATAAGGCCAACAGTC
Solyc04g076660	rgl660	AAGGAGACAGACGATCAAGTAGAGA	ACCGGATCGAAATTCATCACTTGG
Solyc11g011300	rgl11300	TGACAAATCCAACTAACCCAAACCT	AATGCCATTGCTCATCGTCACTTG
Solyc10g049850	TIP41	ATGGAGTTTTTGAGTCTTCTGC	ATGGAGTTTTTGAGTCTTCTGC
Solyc05g006590	PP2Acs	CGATGTGTGATCTCCTATGGTC	AAGCTGATGGGCTCTAGAAATC
Solyc07g064130	UBI	GGACGGACGTACTCTAGCTGAT	AGCTTTCGACCTCAAGGG

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QFG75912.1       1    MR-    -NLKALLLTGIVLCCSSFVDGTAARSPGSHGLPSQenAVEIKMVKNGVVLDNKLVQITFSNPGGDVIAISYGG  74
Solyc04g076660   1    M-G    NGRRFKRMIKCLLCACAGQTGGCCHSGGSGTMSPI--GVRLYIQDRHVMMDNGLVQVTLSNPDGIVTGIRYND  73
Solyc04g076630   1    M-I    VPK---------------------HS----QISSP--VLQLLQHEDQVVIYNGIFQLRLTNPGGKIIGIQYNG  48
Solyc11g011300   1    MRL[12]MGKNMQLISLLYVVEFTMVAFTLLVVDGQRLIESP--IVRLDIQTNQVTMSNGIVNVTLSTPYGFVTSISYGG  86

QFG75912.1       75   IDNLLEINNVEGNRGYWDVVWDNV--AETVHTQNKLPGTQFKVITQSPDQVEISFITTYNASL[4]NGVTVPMNIDKRYI  153
Solyc04g076660   74   VDNLLEGLNDESNRGYWDVVWNSP-DGGKTGVFEVIKGTNFKVIKETDDQVEISFSRPWDPSL   QGKLVPLNIDKRFI  149
Solyc04g076630   49   IENLLELHNPILNGGFWDLNWSVPgTSGTRGKFDEIECNSSKVIVETEEQIELSFIRTWDPSL   QGDQSPLTIDIRYI  125
Solyc11g011300   87   VNNLLATQNVEKDRGYWDIFWNRT---TSKRARKKFLENNFEVIMDTENQAEISFKSTWNAS-   QSDELPLNSDLRFV  159

QFG75912.1       154  LQQGRTGFYTYAIFERLQ--GWPGGRMETLRVAFKLLGAKFRYMAVSDTRQRFMPTAQDRA--RGKPLAYKEAVLMNNeT  229
Solyc04g076660   150  LLRGCSGFYSYAIYEHVGssEWPAFSIGETRIAFKLRKDKFHYMAVADNKQRFMPLPDDRMPARCQRLAYAEAVLLNN-P  228
Solyc04g076630   126  VLRDSPGFYSYAIYEHKE--NMPAFNLNETRIAFMLSFEKFRYMAMADDRQRPMPLPEDRLPPRGKELAYPEAVLLVD-P  202
Solyc11g011300   160  MLRDTPGFYTYAIVERLE--GWPLVYIENLRVVFKLQQDMFHYMAISDERQRIMPMPVDRE--TGKVLDYKEAVLLTN-P  234

QFG75912.1       230  SSPEFSGEVDDKYQYSAEDKDIKVHGWIS-MAPPVGFWMITPSDESRIAGPFKQDLTSHVGPTSMTVFSCSHYVGKEVGL  308
Solyc04g076660   229  VEPELRGEVDDKYQYSCDNKDNKVHGWIS-MDPPVGFWQITPSDEFRSGGPVKQNLTSHVGPTTLAMFLSSHYAGDDLTP  307
Solyc04g076630   203  VEPEFKGEVDDKYQYSCENKDNKVHGFIC-LDPPVGFWQITPSNEFRTGGPIKQDLTSHVNPTTLAMFMSTHYGGQDFVT  281
Solyc11g011300   235  TNPNLKGEVDDKYFYASDNKDDRVHGWVSsTNPPVGFWMIIPNDEFRTGGPYKQDLTSHVGPTVLSIFVSRHFAGDDLVI  314

QFG75912.1       309  KFQEGEAWKKVFGPVFVYLNSAAPSSHSSSMirTLWNNAKDQMLEEVKSWPYNFISSQDYPSSNQRGSVSGQLLVNDPYI  388
Solyc04g076660   308  NFAQGEAWKKVFGPVFIYLNSVMPWED-PV---TLWDDAKIQMQREVQSWPYSFPVSEDFPSADQRGSVSGKLLVQDKYI  383
Solyc04g076630   282  QFGSGEQWKKVFGPVFIYLNSVADKND-AL---SLWYDAKEQMHKEVDCWPYSFASSEDFPKADQRGAIRGRLLVNDRCI  357
Solyc11g011300   315  KFQQGEPWKKVIGPVFLYLNSNSSAMDNPII---LWEDAKRRMNQEVASWPYDFPVSEEYIKSNQRGMVRGQLLINDSSM  391

QFG75912.1       389  PG-IPVASSAYVGLAAPGEVGSWQRESKGYQFWTQTDAKGNFFIEHVRPGHYNLYASVPGIVGDYKYEVDIIIKPGSKIE  467
Solyc04g076660   384  SDDHIPANGAYVGLAPPGEAGSWQKECKDYQFWAKSDNNGNFSINNICPGDYKLYAWVPGFIGDYLYETPISITPGCSID  463
Solyc04g076630   358  SKEYLSAKGAFVGLAPPGDAGSFQRECKGYQFWTNSDDEGYFSIQNVRLGEYNLYAWVPGFIGDYKYEKSIAITAASDVD  437
Solyc11g011300   392  TL--VSASNGYIGLAPPGDVGSWQRENKGYQFWTKTDAMGNFTIENVISGTYNLYATVPGIMGDYKYTFDVQVTPGSSID  469

QFG75912.1       468  LASITYKPPRIGPTLWEIGIPDRSAAEFYIPDPYPTLMNKLYKGSELDKFRQYGLWSRYYELYPHKDLIYNVGANNYGDD  547
Solyc04g076660   464  VDEIVYQPPRDGPTLWEIGIPDRTAAEFFVPEPNPKFVNKLFVNHPD-RFRQYGLWDRYSELYPTDDLVYVTGESDYTKD  542
Solyc04g076630   438  IGELVYEPLRSGPTLWEIGIPDRSAAEFYVPDPDPQYINKLYINQPENRFRQYGLWERYADLYPDQDLVFTIGSSDYKKD  517
Solyc11g011300   470  LGSLVYNPPRNGATLWEIGVPDRTAAEFFVPAPPPEYNVHVYQNNVESIFRQYGLWEQYSILYPENDLVYNVGTSNYSKD  549

QFG75912.1       548  WFYAQVTRSVGNNKYVGTTWQIQFELNAVNP-GIYTLQLALASATYAELEVRVNNANAKPPLFSTGLIGDDNAIARHGIH  626
Solyc04g076660   543  WFFAQVPRRKEDGSYQGTTWQIRFKLDSVNQGGTYKLRIAIASATLAEVQVRVNNPSTNRPVFSTGLIGRDNSIARHGIH  622
Solyc04g076630   518  WFYAQVTRKVGDKAYKPTTWQIKFNLESIDQNGSYTLRIALSSATFSILEVRVNEEEANPPHFSSGLIGSDNTIARHGVH  597
Solyc11g011300   550  WFFAHVTRNIGNKTYISTTWKIIFDLEIVDNASNYTLQIALAAAQGAKLQVRFNDEKVVEPHFTTGKIGGDNAIARHGIH  629

QFG75912.1       627  GLYWFWSIKVPSTLLHKGSNTIYLTQTRGGTPLQGVMYDYIRLEGPAEKP---  676
Solyc04g076660   623  GLYWLYNIDIQGNLLVQGDNTIYLGQPRNQSPFQGIMYDYIRLEAPPN-----  670
Solyc04g076630   598  GLYWLFNVEVAAGQLFQGENIIYLKQARSVSALHGIMYDYIRLEAPPLSTtry  650
Solyc11g011300   630  GLYFLYSIEVKGNLLVNGTNTIFLTQTIATNPFQGVMYDYLRLEGPHQ-----  677
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Figure 1. Amino acid sequence alignment of the three RGL genes. The continuous black line shows the rhamnogalacturonan lyase domain RGL_4N (accession cl15675). The amino acid located at the active site is highlighted in gray. The dotted black line underlines the catalytic site, and the amino acid playing a role in the catalysis of RG-I is highlighted in black. The continuous green line underlines the carbohydrate binding domain RGL4_M (accession cd10316). The continuous red line underlines the carbohydrate binding module (accession pfam14683). The amino acids that bind calcium ions are highlighted in light green.

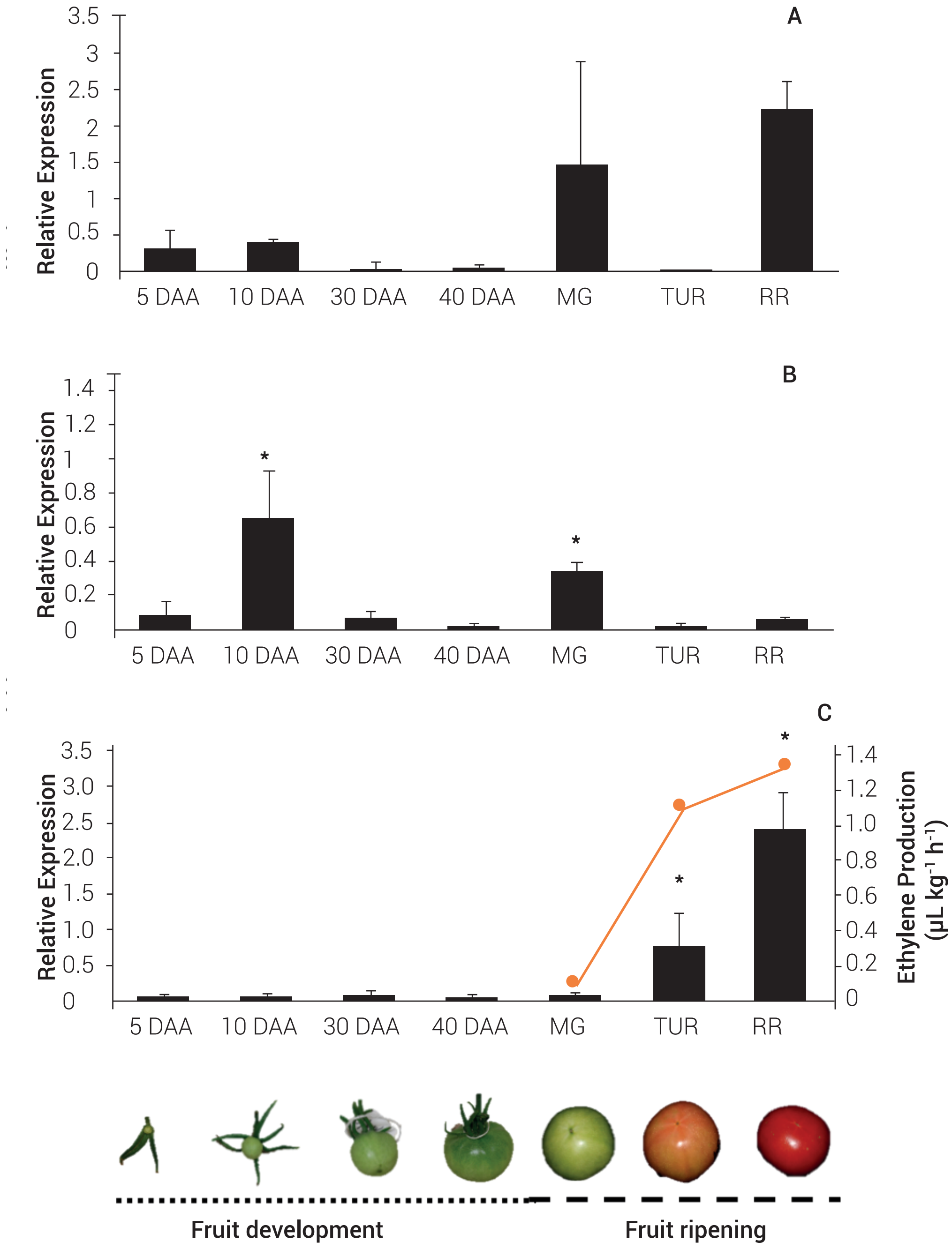

Figure 2. Changes in gene expression of the different rhamnogalacturonan lyase isoenzymes studied through the stages of fruit development and ripening. Panel A shows the relative expression of *Solyc04g076660*, panel B shows the relative expression of *Solyc04g076630* and panel C shows the relative expression of *Solyc11g011300* along with the changes in ethylene production and images of tomato fruits at different DAA. DAA: days after anthesis, MG: mature green, TUR: turning, RR: red ripe. Asterisks on top of the bars indicate statistically significant differences ($P \leq 0.05$). Vertical lines on the bars indicate the standard deviation.

of fruit development at 4 DAA (Pattison *et al.,* 2015), it is feasible to suggest that these genes play a role in tomato fruit growth phenomena by cell division (Azzi *et al*., 2015), although more experimental evidence is needed to further support this statement. Based on results of this study, it is difficult to explain the expression of *Solyc04g076630* in MG and *Solyc04g076660* in MG and RR, although they may play a role during fruit ripening along with the gene *Solyc11g011300*. Studies during the last 20 years had shown the coordinated expression of several gene families acting over the PCW, such as peroxidases (Andrews *et al.,* 2000), polygalacturonase, pectate lyase (Crookes and Grierson, 1983) and pectin methyl esterase (Giovane *et al.,* 1993); the combined action of these gene families results in complex changes during fruit ripening phenomena (Rivera-Domínguez *et al*., 2008).

The expression of an RGL gene during ripening was reported in two species of strawberry, *Fragaria × ananassa* (Molina-Hidalgo *et al*., 2013) and *Fragaria chiloensis* L. (Méndez-Yañez *et al.,* 2020) . Additionally, the role of the RGL gene in fruit strawberry during softening was demonstrated by linkage association. Sequence comparison between tomato *Solyc11g011300* and the strawberry rhamnogalacturonan lyase gene (*FaRGLyase1*) resulted in 65 % identity (Berumen-Varela *et al*., 2018). In this experiment, high expression of the *Solyc11g011300* gene was found during fruit ripening (Figure 2C), similar to the expression of the *FaRGLyase1* gene (Molina-Hidalgo *et al.,* 2013). Based on the above mentioned findings, it is possible to suggest that the *Solyc11g011300* gene from tomato plays a role in fruit softening, although more experimental evidence is needed to support this statement.

Figure 2C also shows the changes in ethylene production. In the MG stage, ethylene production of 0.13 mL kg^{-1} h^{-1} was recorded, but ethylene was increased 10-fold (1.31 mL kg^{-1} h^{-1}) in the RR stage relative to the MG stage. It was found that *Solyc11g011300* gene expression and ethylene production showed a similar trend during fruit ripening, with a positive correlation of 0.9, which is rather high. The accumulation of *Solyc11g011300* transcripts and ethylene production begin at the same time, maintaining a large positive correlation during fruit ripening. In addition, the ethylene responsive element ERELE4 was found in the *Solyc11g011300* promoter (Berumen-Varela *et al.*, 2017). In climacteric fruits, ethylene is responsible for the activation of genes that induce color changes, alter starch-sugar metabolism, soften fruits and synthesize volatiles related to aroma, among other changes. In this regard, the *Solyc11g011300* gene is probably one of the genes that are activated by ethylene to carry out the changes in texture and firmness observed in fruits during ripening, although more experimental evidence is needed to support this statement.

CONCLUSION

The tomato RGL genes *Solyc11g011300*, *Solyc04g076660* and *Solyc04g076630* show low similarity with the strawberry rhamnogalacturonan lyase gene (QFG75912.1); however, they do contain the three structural domains found in other rhamnogalacturonan lyase enzymes, including the catalytic amino acids. It is suggested that the *Solyc04g076660* and *Solyc04g076630* genes could be involved in carrying out changes to RG-I during the early stages of tomato fruit development, in which the fruit is growing mainly by cell expansion. On the other hand, the *Solyc11g011300* gene could play a role in fruit softening during fruit ripening.

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