Overexpression of glycine-rich RNA-binding protein in tomato renders fruits with higher protein content after cold storage

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Abstract

Glycine-rich RNA-binding proteins (GR-RBPs) are involved in RNA processing and also some of them are output signals of the circadian clock. In tomato, one *GR-RBP* gene family (*LeGRP1*) is composed by three highly homologous genes (*LeGRP1a-c*); each one rendering three transcriptional products: the un-spliced pre-RNA (*preLegrp1a-c*), the mature mRNA (*mLegrp1a-c*) and the alternatively spliced mRNA (*asLegrp1a-c*). To get insight into their regulation and impact on RNA metabolism in fruits, *Solanum lycopersicum* cv. Micro-Tom was transformed with *preLeGRP1a* fused to the polygalacturonase promoter, which drives expression to fruits from the mature green stage. Our results demonstrated a complex positive regulation of *LeGRPs*, in which *LeGRP1a* overexpression led to the induction of the others *LeGRP1* members. Even though the *LeGRP1* transcription and the content of three LeGRPs proteins were affected, the overall LeGRP protein circadian rhythm profile was similar in transgenic and WT fruits. However, when the fruits are kept at chilling temperature after harvest, total protein content was significantly higher in transgenic than in WT fruits, and the content of some free amino acids was modified. The results obtained suggest a probable role of LeGRP1s: structural rearrangements and/or stabilization of mRNA to allow efficient processing of fruits under cold conditions.

Additional key words: amino acids, circadian rhythm, polygalacturonase promoter, RNA stabilization.

Introduction

In order to respond and adapt to the changing environment, living organisms regulate gene expression. Transcriptional and post-transcriptional control of RNA content as well as RNA splicing, capping, polyadenylation, transport, stability and translation, have important roles in growth, development, and in the response to numerous stresses (Lorković 2009, Jung *et al*. 2013). RNA binding proteins (RBPs) are proteins that bind to RNA molecules enabling its processing and metabolism, such as splicing, polyadenylation, editing, stabilization, localization, and translation. At low temperatures, RBPs are essential, because RNA molecules adopt misfolded-stabilized structures and RBPs allow them to acquire native conformation (Phadtare 2011, Kang *et al*. 2013).

Plants genomes encode hundreds of RBPs (Cook *et al*. 2011). Among these proteins, glycine-rich RNA-binding proteins (GR-RBPs) function as RNA chaperones during growth, development, and under stress conditions (Lorković 2009, Kim *et al*. 2010, 2012, Kang *et al*. 2013, Ambrosone *et al*. 2015). GR-RBPs contain an *N*-terminal RNA recognition motif (RRM) domain involved in RNA recognition and a glycine-rich (GR) *C*-terminal domain implicated in protein-protein interactions (Sachetto-Martins *et al*. 2000). Plant GR-RBPs were mainly studied in *Arabidopsis thaliana* and *Oryza sativa.* Over-

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Abbreviations: CI - chilling injury; CSD - cold shock domain; DAA - days after anthesis; GR-RBPs - glycine-rich RNA-binding proteins; IG - immature green; MG - mature green; PG - polygalacturonase; PITC - phenylisothiocyanate; RT-qPCR - reverse transcription - quantitative polymerase chain reaction; RBP - RNA binding protein; RR - red ripe; RRM - RNA recognition motif; TEA - triethylamine; WT - wild-type; Y - green yellowish.
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expression of GR-RBPs in *Arabidopsis* enhances its tolerance to salinity, cold, and oxidative stress (Kim *et al*. 2007a,b, 2008, Sahi *et al*. 2007, Ayarpadikannan *et al*. 2014, Tan *et al*. 2014). However, to our knowledge, no studies analyzing GR-RBPs overexpression in fruits were performed.

In our previous work, a GR-RBP family, which was called *LeGRP1*, was characterized in tomato fruits (Müller *et al*. 2014). Three highly homologous *LeGRP1* genes (*LeGRP1a-c*) were identified. For each gene, three transcriptional products have been found, corresponding to the un-spliced pre-RNA, the mature mRNA, and the alternatively spliced mRNA (*preLeGRP1a-c*, *mLeGRP1a-c* and *asLeGRP1a-c*, respectively). LeGRP1s harbored *in vivo* RNA-melting abilities, and complemented the cold-sensitive phenotype of a particular *Escherichia coli* strain. Differential circadian profiles of expression, dependent on the fruit developmental stage, have been found for each *LeGRP1* form. During ripening of fruits harvested at the mature green (MG) stage off the vine, the content of all *Legrp1a-c* forms increases considerably; however, incubation at 4 ºC prevents such increase (Müller *et al*. 2014).

Tomato is one of the most important vegetable crops. Commercial tomatoes are subjected to a postharvest handling system that chills fruit to extent shelf life (Lana *et al*. 2005). Low-temperature storage is widely used to slow ripening and reduce decay. However, the fruits are sensitive to chilling injury (CI) at temperatures below 10 ºC (Jackman *et al*. 1988, López Camelo 2004, Zhang *et al*. 2016).

Considering the role of GR-RBPs in RNA metabolism during stress conditions such as cold (Kim *et al*. 2010, Kwak *et al*. 2011, Sasaki and Imai 2012), the working hypothesis is that GR-RBP overexpression in tomato may modify fruit quality by inducing RNA structural rearrangements and/or processing during postharvest cold storage. To test this hypothesis, *Solanum lycopersicum* cv. Micro-Tom was transformed with the complete sequence of the *preLeGRP1a* fused to the poly-galacturonase promoter, which drives expression in fruits from the mature green (MG) stage (Lau *et al*. 2009). The effect of *LeGRP1a* overexpression on circadian oscillation of all LeGRPs family members was analysed. In addition, total protein content and content of some free amino acids was compared in fruits of transgenic and wild-type (WT) tomato after cold storage.

Materials and methods

Plasmid construct and plant transformation: A 4.8 kbp promoter region of the *Solanum lycopersicum* Mill. polygalacturonase gene (*PG*, Gene Bank: FJ465170) and the full-length sequence of the *preLegrp1a* cDNA (*preLegrp1a*, Gene Bank: FJ465170) were cloned between the NotI and PstI restriction sites of a modified version of the pGreen II vector (Fahnenstich *et al*. 2007) bearing the kanamycin resistance gene. Full length of PG promoter (Lau *et al*. 2009) was obtained through PCR using genomic DNA from leaves of tomato cv. Micro-Tom (PG1F 5'-GCGGCCGCCCTGCAGGTC AACGG-3' and PG3R 5'-GGTACCTAGTCTATT GAAAAAGATTATT-3') and the full-length sequence of the *preLegrp1a* was achieved from PCR using immature green (IG) fruit cDNA (GRP1aF 5'-GGATCCTTA GGGTTTCTCTTTTCTGG-3' and GRP1aR 5'-CTG CAGACATGCTTTTACTTGATTCC-3').

Agrobacterium tumefaciens (strain GV3101) containing the "helper" pSOUP plasmid was transformed with the *pGreenII::PG::preLeGRP1a* construct by the freeze thaw method (An *et al*. 1988). Thus, *S. lycopersicum* cv. Micro-Tom *PG::preLeGRP1a* transgenic lines were generated by *Agrobacterium*-mediated transformation (Fernandez *et al*. 2009). When the plantlets have a root of 2 cm length, they were transferred to soil. After several rounds of selection on plates containing 100 mg dm^{-3} kanamycin, homozygous lines were obtained (*PG::preLeGRP1a* plants) and seeds were collected.

Plants and storage conditions: *S. lycopersicum* WT and *PG::preLeGRP1a* transgenic lines were grown from seeds as described in Müller *et al*. (2014). Flowers were tagged after pollination and fruits were allowed to develop until different stages according to the number of days after anthesis (DAA) and harvested for analysis or used for the postharvest treatments.

Transgenic tomatoes (*PG::preLeGRP1a*) were harvested at the immature green (IG, 16 DAA) and red ripe (RR, 47 DAA) stages. For time course analysis, IG and RR fruits were harvested from the vine grown in 16-h photoperiod at 4 h intervals (zeitgeber time - zt 0, 4, 8, 12, 16, 20). In addition, *PG::preLeGRP1a* tomato plants with IG or RR fruits grown under a 16-h photoperiod were transferred to continuous irradiance for 24 h and fruits were harvested at 4 h intervals (zeitgeber time ztcl 4, 8, 12, 16, 20, and 24; Fig. 1 Suppl.). Therefore, the expression analysis was done comparing fruits subjected to 16-h photoperiod with those exposed to continuous irradiance. The following comparisons were conducted: ztcl4 with zt20, ztcl8 with zt0, ztcl12 with zt4, ztcl16 with zt8, and ztcl24 with zt16 (Fig. 1 Suppl.). Time course analyses were performed on fruits collected from different plants and repeated at least three times.

To analyze the effect of *preLegrp1a* overexpression during storage at chilling temperatures, WT and transgenic *PG::preLeGRP1a* fruits were harvested at green yellowish stage (Y; 38 - 40 DPA, first visible carotenoid accumulation) and stored at 4 °C for 7 d (7D4, Fig. 2 Suppl.). Then, the fruits were transferred to 20 ºC for several days, each day was named as +1, +2, +3, +4 in fruits 7D4 (7D4+1, +2, +3, +4; respectively; Fig. 2

Suppl.). Each treatment was performed with at least five fruits and was repeated at least five times.

At each sampling point, fruits were cut into pieces; pulp and seeds were removed and the remaining pericarp was frozen in liquid nitrogen and stored at -80ºC for extraction of chlorophyll, lycopene, protein, and RNA.

Firmness, coloration, and fresh mass were determined according to Müller *et al*. (2013). Percentage of soluble solids in tomato juice was tested using a refractometer *Atago N1* for 0 - 32 °C (USA; Borsani *et al*. 2009). Dehydration was visually evaluated using the following scale: 0 - 20 % = no dehydration, 1 = 20 - 60 % of the fruit visibly dehydrated, 2 = more than 60 % of the fruit dehydrated. Total chlorophyll and carotenoids were extracted and quantified according to Nagata and Yamashita (1992).

Protein extraction and quantitation, SDS-PAGE and Western blot analysis: Total soluble proteins from the different samples were extracted as previously described by Müller *et al*. (2014). Protein content was determined by the method of Bradford (1976) using the *Bio-Rad* (Hercules, CA, USA) protein assay reagent and bovine serum albumin as standard. Coomassie blue stained SDS-PAGE was conducted for protein loading control before Western blot analysis (Fig. 3 Suppl.). SDS-PAGE was carried out using 15 % (m/v) polyacrylamide gels according to Laemmli (1970). Proteins were visualized with Coomassie blue or electroblotted onto a nitrocellulose membrane for immunoblotting according to Burnette (1981). Bound antibodies were located by linking to alkaline phosphatase-conjugated goat anti-rabbit IgG according to the manufacturer's instructions (*Bio-Rad*). Purified antibodies raised against LeGRP1a protein (1:1000) were used (Müller *et al*. 2014). These antibodies detect LeGRP1a, LeGRP1b, and LeGRP1c proteins corresponding to 16 and 17 kDa immunoreactive bands (Müller *et al*. 2014). Immunoreactive 16 and 17 kDa bands were semi-quantified by densitometric analysis. For this, images were scanned and the area of the bands was integrated by use of a *BioChemi System UVP* (*BioImaging System*, Upland, CA, USA). In integrating peak areas, a rectangular bar covering ~80 % of each lane was drawn. Each densitometric analysis was determined in triplicate to minimize experimental errors.

RNA isolation, reverse transcription (RT)-PCR, and quantitative real-time PCR: Total RNA, RT-PCR and quantitative real-time PCR were previously described in Müller *et al.* (2014). Oligonucleotides primers are listed in Table 1 Suppl. Each pair of primers detects each isoform specifically as it was reported in Müller *et al*. (2014).

Free amino acid analysis: The amino acid content of tomato extracts was determined using reverse phase-HPLC with pre-column phenylisothiocyanate (PITC) derivatization (Dhar *et al*. 2013). The reverse phase C18 column (5 µm, 250 × 4.6 mm, *LUNA Phenomenex*, USA) with a C18 guard security pre-column (4 × 3 mm) was connected to *ÄKTA* purifier equipment (*GE Healthcare*, Uppsala, Sweden). Individual 23 amino acid standards (cysteine, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine, threonine, valine, alanine, aspartic acid, glutamic acid, glycine, proline, serine, asparagine, glutamine, cystine, ornithine, citrulline, and tryptophan; 50 µM) were prepared in distilled water or 0.1 M HCl in accordance with their solubility. A working standard mixture was prepared by diluting the intermediate stock standard solution to 2 µM concentration.

Calibration curves were done in the range 0.2 - 20 nM of each amino acid and repeated two times using two different working standard amino acids mixtures. The derivatization procedure was as follows: an equal volume of coupling reagent [methanol/ water/triethylamine (TEA); 2:2:1, v/v] was added to each point of the curve and the solution was mixed and dried immediately under vacuum. After this, PITC reagent (methanol/TEA/water/ PITC, 7:1:1:1, v/v) was added and allowed to stand at room temperature for 20 min before drying in vacuum. PITC derivatives were dissolved in 0.5 cm^3 of sodium acetate buffer (mobile phase A). For mobile phase A, 19 g of sodium acetate trihydrate was dissolved in 1 dm^3 of HPLC grade water. To this, 0.5 cm^3 of triethylamine (TEA) was added and the content was mixed properly, adjusted to pH 6.4 with glacial acetic acid, and filtered. Acetonitrile (60 cm^3) was added to the filtrate (940 cm^3), mixed and filtered through a 0.22 µm *Millipore* (Darmstadt, Germany) membrane.

The injection volume of the sample and standard was 0.1 cm^3. Separation of amino acids was performed on reverse phase C18 column with sodium acetate buffer, pH 6.4 (mobile phase A) and acetonitrile/H_2O (6:4, mobile phase B) with gradient mode of operation (Table 2 Suppl.), column temperature of 39 ºC, and 150 kPa limit of pressure. The absorbance at 254 nm was recorded and used for calculation. Chromatogram resolution of the amino acids standards and calibration curves are shown in Figs 4 and 5 Suppl.

For analysis of free amino acids in tomato fruits, pieces of fruits (approx. 0.3 g) were processed using a mortar and pestle, and 1 cm^3 of 0.1 M HCl. After centrifugation at 14 000 *g* and 4 ºC for 15 min, the supernatant was precipitated with 10 % (v/v) trichloroacetic acid (TCA) and maintained on ice for 30 min. After centrifugation at 14 000 *g* and 4 ºC for 15 min, 0.05 cm^3 of the supernatant was used for derivatization using the procedure described above. PITC derivatives were dissolved in 0.3 cm^3 of sodium acetate buffer (mobile phase A). All the volumes were documented and each amino acid was quantified using the calibration curve (Table 3 Suppl.).

Statistical analysis: Data from the qPCR experiments were tested using one-way analysis of variance (*ANOVA*). Continuous quantitative variables such as mass, firmness, and soluble solids were analyzed using two-way *ANOVA*.

Protein content was assessed taking in account variability between harvests, testing the three harvests pairwise alone using two-way *ANOVA*. When error normality assumption was not verified, logarithmic transformation was applied. The absence of interaction between variables was confirmed in all cases. Minimum significance differences were calculated by the Bonferroni, Holm-Sidak, Dunett, and Duncan tests ($P = 0.05$) using the *Sigma Stat Package*. Also Spearman rank order correlation analysis was conducted with the *Sigma Stat Package*. Dehydration grade was analyzed using Score testing.

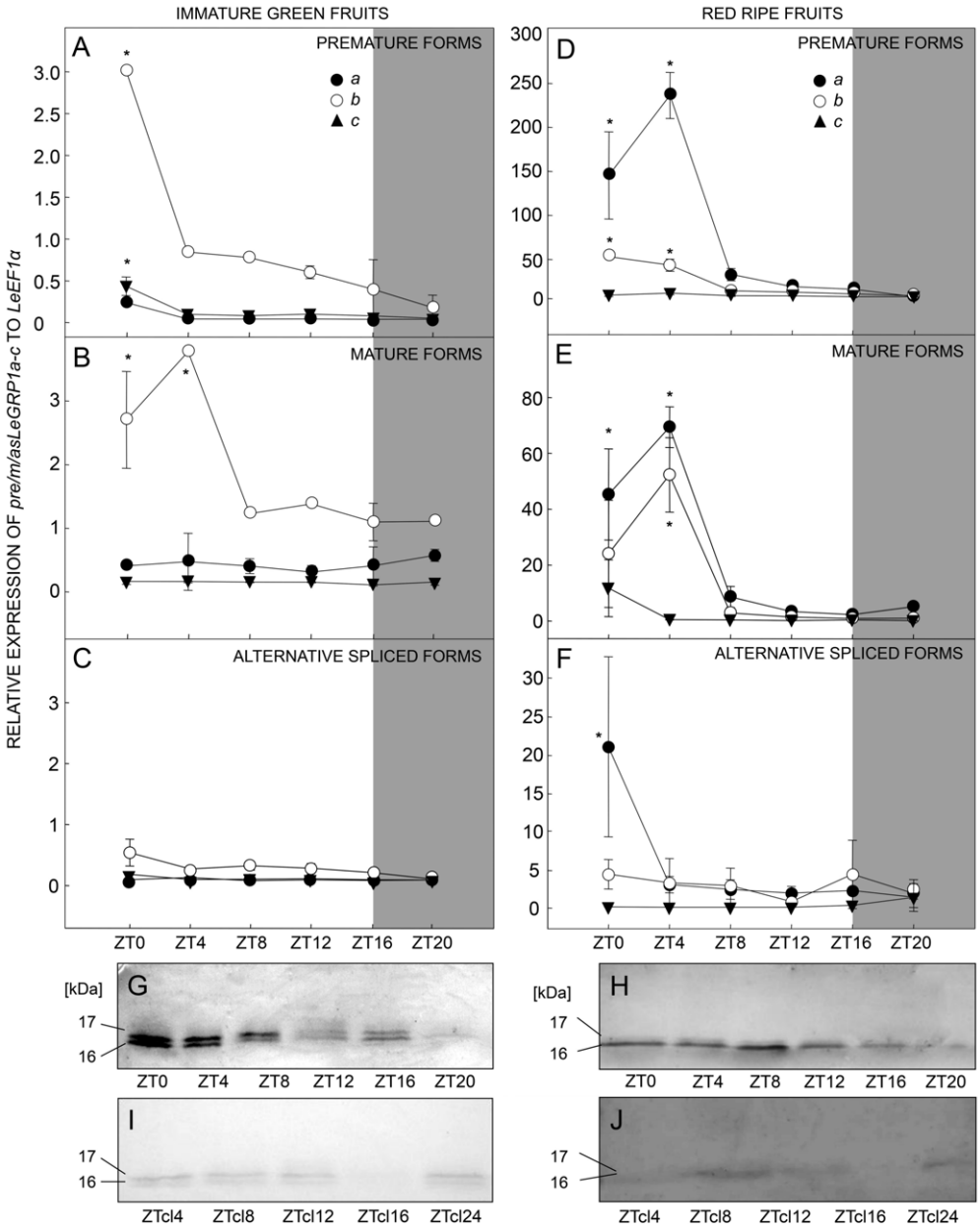

Fig. 1. *LeGRP1a-c* premature, mature, and alternative spliced mRNA (*A-F*) and LeGRP1 protein content (*G-J*) in transgenic *PG::preLeGRP1a* fruits at the immature green (IG, *left panel*) and red ripe (RR, *right panel*) stages. Fruits of plants grown under 16-h photoperiod were harvested at 4-h intervals. Means ± SDs of the results obtained by real-time quantitative PCR, using two independent mRNAs as templates are shown. The y-axis refers to the fold difference in transcription relative to the *LeEF1α* level. Light and dark periods are represented by *white* and *grey* areas, respectively; and the Zeitgeber time (Zt) is indicated in the graph. For each transcript, *asterisks* indicate that the values are significantly different ($P < 0.05$). Western blot using 25 µg (*G* and *I*) and 10 µg (*H* and *J*) of pericarp protein extracts were revealed with antibodies raised against LeGRP1a. Immunoreactive bands of 16 and 17 kDa are shown. Plants with IG (*I*) or RR (*J*) fruits were transferred to continuous irradiance for a 24 h and fruits were harvested at 4-hourly intervals (zeitgeber time - ztcl 4, 8, 12, 16, and 24; Suppl. Fig. 1). One representative image of three replicates is shown.

Results

To increase the content of LeGRP1a protein in tomato fruits, we integrated the full-length sequence of the premature *LeGRP1a* cDNA (preLegrp1a, Solyc10g051390) under the control of tomato PG promoter (Gene Bank: FJ465170, which drives high expression in tomato fruit from the mature green stage), into the *S. lycopersicum* cv. Micro-Tom genome. Two independent *S. lycoperisucum* *PG::preLeGRP1a* transgenic homozygous lines from the third generation (lines 1 and 2) containing the transgene were selected for further analysis.

Under normal growing conditions, no significant phenotypic changes were observed among WT and the two transgenic lines (Fig. 6 Suppl.); they were between 25 to 30 cm high, and they had similar flowering time. Mature green (MG) and red ripe (RR) fruits were obtained at 36 - 38 and 45 - 48 DAA, respectively. The diameter of RR fruits was also similar (2 - 2.3 cm). Since the expression of LeGRP1a was directed to the fruits, we focused on the analysis of proteins in this organ at different developmental stages.

Time-course of all *LeGRP1a-c* transcriptional forms and LeGRP1 protein was analyzed over a 24-h period in fruits of *PG::preLeGRP1a* lines by real time qPCR and Western blot at two developmental stages: immature green (IG) and RR (Fig. 1).

Transgenic fruits displayed a similar pattern of time-course fluctuation of the premature forms of *LeGRP1*, *preLeGRP1a* and *c* at IG and *preLeGRP1a* and *b* at RR stage, with the greatest expression at the beginning of the

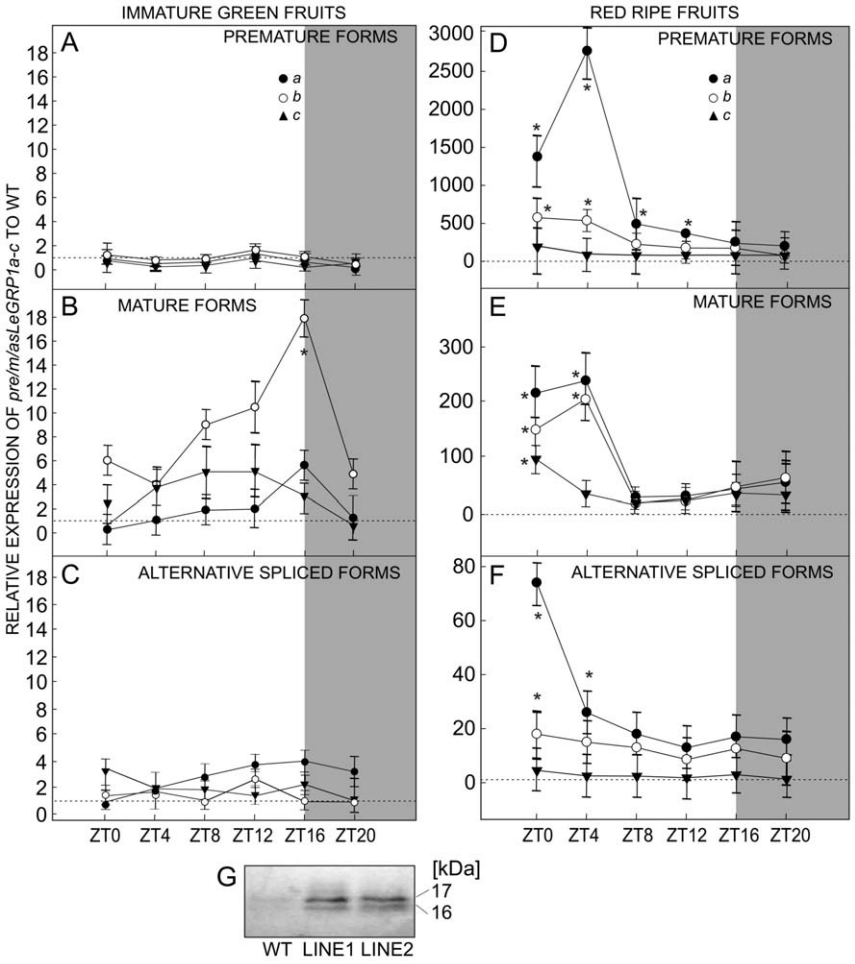

Fig. 2. *LeGRP1a–c* premature, mature, and alternative spliced mRNA content in *PG::preLEGRP1a* relative to WT fruits. Fruits of transgenic *PG::preLEGRP1a* plants were harvested at the immature green (IG, *left panel*) and red ripe (RR, *right panel*) stages from plants grown at a 16-h photoperiod at 4-h time intervals. Light and dark periods are represented by *white* and *grey areas*, respectively; the Zeitgeber time (Zt) is indicated on the x-axes. Means ± SDs of the results obtained using two independent mRNAs as templates. The y-axis refers to the fold difference in a particular transcript level in *PG::preLEGRP1a* relative to its level in WT (*dashed lines*). For each transcript, *asterisks* indicate that the values are significantly different from WT ($P < 0.05$). *G* - Western blot using 15 µg of RR pericarp protein extracted from fruits at zt0 was revealed with antibodies raised against LeGRP1a. Immunoreactive bands of 16 and 17 kDa are shown. One representative image of three replicates is shown.

day light (zt0 - zt4; Fig. 1*A,D*). In accordance, the mature forms, *mLeGRP1b* at IG and *mLeGRP1a* and *b* at RR stage, peaked at the first hours of irradiance (Fig. 1*B,E*). Regarding the alternatively spliced forms, *asLeGRP1a-c* exhibited little variation along the time at IG stage (Fig. 1*C*) but at the RR stage, *asLegrp1a* showed the highest content at zt0 (Fig. 1*F*). The relative expression of each *LeGRP1* transcript in *PG::preLeGRP1a* lines with respect to the levels found in WT (Müller *et al*. 2014) is shown in Fig. 2. At IG stage, no significant modifications in transcription were detected in the *PG::preLeGRP1a* lines in comparison with the WT, albeit the increase of *mLeGRP1b* expression at zt16 was found (Fig. 2). In contrast, at the RR stage drastic increases in expression were detected (Fig. 2), which agrees with the reported activity of the PG promoter. Particularly, the content of *preLeGRP1a* increased nearly 2 800 times at zt4 in RR transgenic fruits with respect to that found in WT (Fig. 2*D*). In RR fruits, not only the *preLeGRP1a* content was increased, but also the content of its mature (*mLegrp1a*) and alternatively spliced (*asLeGRP1a*) forms, which increased at zt0 nearly 200 and 80 times, respectively (Fig. 2*E,F*). Surprisingly, the amount of the transcriptional forms of *LeGRP1b* and *LeGRP1c* were also drastically increased in relation to WT fruits at RR: nearly 500 times for *preLeGRP1b* at zt0 - 4, 180 times for *mLeGRP1b* at zt4, 20 times for *asLeGRP1b* at zt0 and 90 times for *mLeGRP1c* at zt0 (Fig. 2*E,F*.). Accordingly to the content of the mature forms in transgenic RR fruits, the amount of the immunoreactive LeGRP1 bands increased with respect to WT (nearly 8 times, Fig. 2*G*). In transgenic *PG::preLeGRP1a*, at IG stage the highest content of immunoreactive protein LeGRP1 occur concomitantly with the highest amount of the mature *LeGRP1b* form (Fig. 1*G*) and similarly to the profile found in WT fruits. In contrast, at RR, the highest content of immunoreactive LeGRP1 protein was observed at zt8 (Fig. 1*H*). This result contrasts with the pattern of expression of the transcriptional mature forms, which peak earlier than zt8 (Fig. 1*E*), but it agrees with the pattern detected in WT (Fig. 1*H,* Müller *et al.* 2014). Additionally, when transgenic plants with fruits at IG or RR stages, grown under a 16-h photoperiod, were transferred to continuous irradiance for 24 h, the patterns of accumulation of LeGRP1 protein remained practically the same when compared to fruits under a 16-h photoperiod (Fig. 1*I,J*).

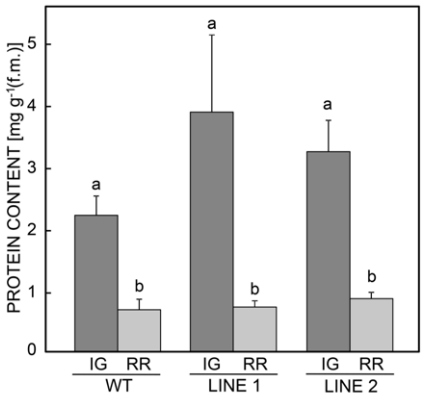

Fig. 3. Total protein content of WT and *PG::preLEGRP1a* fruits in IG and RR stages. Means ± SDs, n = 30 Bars with the same letters are not significantly different ($p < 0.05$).

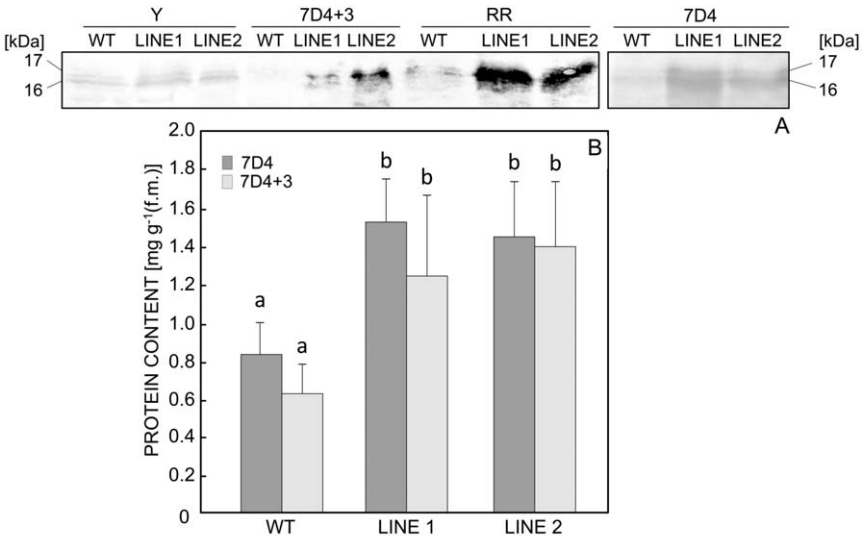

Fig. 4. Effects of cold treatments on the protein content of WT and *PG::preLeGRP1a* fruits. Fruits were harvested at green yellowish (Y) or RR stage. Y fruits were incubated at 4 ºC for 7 d (7D4) and then transferred to 20 ºC for another 3 d (7D4+3). *A* - Pericarp protein extracts (25 µg) were loaded in each lane and were revealed using antibodies against LeGRP1a. Immunoreactive bands of 16 and 17 kDa are shown. One representative image of at least three replicates is shown. *B* - Protein content of WT and *PG::preLeGRP1a* fruits after storage for 7 d at 4 ºC (7D4) and after further ripening (3 d at 20 ºC, 7D4+3). Means ± SDs, $n = 30$. The experiment was repeated in three consecutive harvests. *Bars* with the same letters are not significantly different among the treatments ($P < 0.05$).

Further we tested whether the overexpression of *LeGRP1*a has an impact on the protein content in IG and RR fruits from transgenic lines. Independently of the genotype, the protein content was significantly higher in IG than in RR fruits (Fig. 3). However, no significant differences in total protein content were detected between fruits from WT and from transgenic lines at both IR and RR stages (Fig. 3).

In order to address the response of transgenic fruits to cold storage, fruits from *PG::preLeGRP1a* transgenic lines and WT were harvested at green yellowish stage (Y) and stored at 4 ºC for 7 d (7D4) and then kept at 20 ºC for 3 days to allow ripening (7D4+3, Fig. 2 Suppl.). Fruit firmness, coloration, mass, and soluble solids of 7D4+3 WT and transgenic fruits are summarized in Table 1. No significant differences in these parameters were detected between WT and transgenic fruits. Quality parameters and visual inspection of the fruits indicate that the treatment explored in this work (7 days at 4ºC) does not affect the subsequent ripening of the fruits when transferred to 20 ºC (Table 1 and Fig. 2 Suppl.). Accordingly, the content of pigments (chlorophylls and lycopenes) was similar in WT and *PG::preLeGRP1a* fruits (Fig. 7 Suppl.). In addition, no differences in visual dehydration were observed (Fig. 7 Suppl.).

Table 1. Fresh mass, firmness, total soluble solids, and coloration of WT and *PG::LeGRP1a* fruits (lines 1 and 2) after cold treatment at 4 ºC for 7 d and subsequent storage at 20 ºC for 3 d. Fruit colour was monitored using the *CIE L*a*b* (L: lightness, a: green-to-red scale, b: blue-to yellow scale). Firmness is expressed in Newton (N). Means ± SDs, $n = 30$. Values with an identical letter are not significantly different ($P < 0.05$).

	Fresh mass [g]	Firmness [N]	Soluble solids [% of f.m.]	Colour L	a*	b*
WT	1.8±0.2 [a]	63.4±4.0 [a]	5.0±0.4 [a]	53.9±1.5 [a]	27.5±2.1 [a]	44.1±1.9 [a]
Line 1	1.6±0.1 [a]	67.9±2.7 [a]	5.2±0.2 [a]	53.3±1.5 [a]	27.6±1.4 [a]	44.0±1.3 [a]
Line 2	1.7±0.1 [a]	69.4±2.8 [a]	5.2±0.2 [a]	55.2±1.1 [a]	25.4±1.4 [a]	46.7±1.3 [a]

Table 2. The content of free amino acids [µmol g^{-1}(f.m.)] in WT and transgenic fruits (nd - not detected). Means ± SDs, $n = 6$. Values with different letters are significantly different ($P < 0.05$).

Amino acids	WT	Line 1	Line 2
Asp	1.90±0.30 [a]	0.90±0.40 [a]	1.31±0.30 [a]
Glu	0.31±0.01 [a]	0.27±0.08 [a]	0.40±0.10 [a]
Asn	0.40±0.20 [a]	0.50±0.10 [a]	0.33±0.07 [a]
Ser	0.40±0.20 [a]	0.15±0.10 [a]	0.33±0.07 [a]
Gln	0.60±0.20 [a]	0.40±0.20 [a]	0.50±0.10 [a]
Gly	nd	0.80±0.10 [a]	1.00±0.20 [a]
His	0.13±0.06 [a]	0.13±0.03 [a]	0.29±0.05 [a]
Citrulline	0.04±0.01 [a]	0.11±0.03 [a]	0.09±0.03 [a]
Arg	0.20±0.08 [a]	0.11±0.03 [a]	0.18±0.05 [a]
Ala	0.30±0.10 [a]	0.30±0.10 [a]	0.40±0.10 [a]
Pro	0.80±0.10 [a]	1.00±0.30 [a]	1.00±0.30 [a]
Tyr	0.01±0.05 [a]	0.06±0.03 [a]	0.10±0.30 [a]
Val	0.40±0.05 [a]	0.30±0.10 [a]	0.30±0.07 [a]
Cystine	0.16±0.02 [a]	0.16±0.03 [a]	0.19±0.03 [a]
Orn	0.62±0.05 [a]	0.36±0.05 [b]	0.20±0.04 [b]
Lys	nd	0.19±0.03 [a]	0.18±0.03 [a]

The content of LeGRP1 protein was higher in the transgenic *PG::preLeGRP1a* lines than in WT after the fruits were exposed to cold treatment and after subsequent ripening (7 times in average, Fig. 4*A*). However, these amounts were lower than when transgenic fruits ripened on the plant (2.7 times in average, RR fruits, Fig. 4*A*).

Total soluble protein content was analyzed in transgenic and WT fruits. Higher protein content in transgenic than in WT fruits (average 50 % higher) was determined after cold storage (7D4), and after subsequent ripening at 20 ºC (7D4+3, Fig. 4*B*, Fig. 8 Suppl.). The overall pattern of protein bands, assessed by SDS-PAGE, was similar in transgenic and WT fruits (Fig. 8 Suppl.).

Considering the difference in protein content, free amino acids in fruits after cold treatment were also investigated (Table 2). The relative amounts of the majority of the amino acids (Asp, Glu, Asn, Citrulline, Val, Gln, His, Ser, Arg and Cystine) did not change in the transgenic fruits in comparison to the WT. On the other hand, the content of Orn was higher in WT than in transgenic fruits. Notably, Lys and Gly were found in fruits from transgenic lines but not in those from WT plants (Table 2).

Discussion

In agreement with high PG promoter activity at RR stage, fruits from *PG::preLeGRP1a* plants showed high expression of *preLeGRP1a* and the highest one occurring during the first hours of irradiance (Fig. 1*D* and Fig. 2*D*). In addition to the great increase in *preLeGRP1a* expression, *mLeGRP1a* expression was also higher in

transgenic than in WT plants (Fig. 2*D-E*), demonstrating that *preLeGRP1a* transcription was correctly processed in respective fruits. Interestingly, all transcript forms of *Legrp1b* gene were also increased in transgenic fruits with respect to WT fruits in RR stage (Fig. 2*D-F*). The increase in transcription occurred concomitantly with a significant increase in LeGRP1 protein content (Figs. 2 and 4). Although the increase of *Legrp1a* and *-b* expression and LeGRP1 protein content in transgenic fruits with respect to WT was found, the circadian profile of LeGRP1 protein in fruits at RR stage was similar to that observed in WT, with maximum at zt8 in RR (Fig. 1*H*; Müller *et al*. 2014). In accordance with the high expression of *mLeGRP1a-c* in transgenic *PG::preLeGRP1a* RR fruits (Figs. 1*E* and 2*E*), the content of LeGRP1 protein in RR *PG::preLeGRP1a* fruits was 8 times higher than in WT RR fruits (Fig. 2*G*).

Overall, the results obtained are in accord with a positive regulation of the different LeGRPs in tomato fruit (Fig. 9 Suppl.). The overexpression of *preLeGRP1a* under the control of PG promoter in RR fruits generated the accumulation of *preLeGRP1a,* which was processed to the mature form (*mLeGRP1a*) that led to an increase in LeGRP1a protein. This protein would activate the expression of *Legrp1b-c* genes through an unknown mechanism. In turn, immature transcripts *preLeGRP1b* and *c* would be processed to their mature forms (*mLeGRP1b* and *c*, respectively) and finally, they could also be translated; contributing to the increase of LeGRP1 protein (Fig 2*D-G* and Fig. 4*A*). Moreover, these proteins could, in turn, regulate *Legrp1a-c* genes. This model, based on results obtained here, deeply differs from the one proposed for *A. thaliana* leaves, in which *AtGRP7* negatively regulates the expression of *AtGRP8* and *vice versa* (Schöning *et al*. 2007, 2008, Staiger *et al*. 2003). In the case of tomato fruits, the regulation would also be reciprocal, but in contrast to the *Arabidopsis* model, it would be positive since the increase of *LeGRP1a* promotes the increase of *LeGRP1b* and *c*. The model presented here is in accordance with the transcript correlation analysis proposed for tomato WT RR fruits, in which *preLeGRP1a-c* transcripts positively correlate with each other and with *mLeGRP1a* (Müller *et al*. 2014).

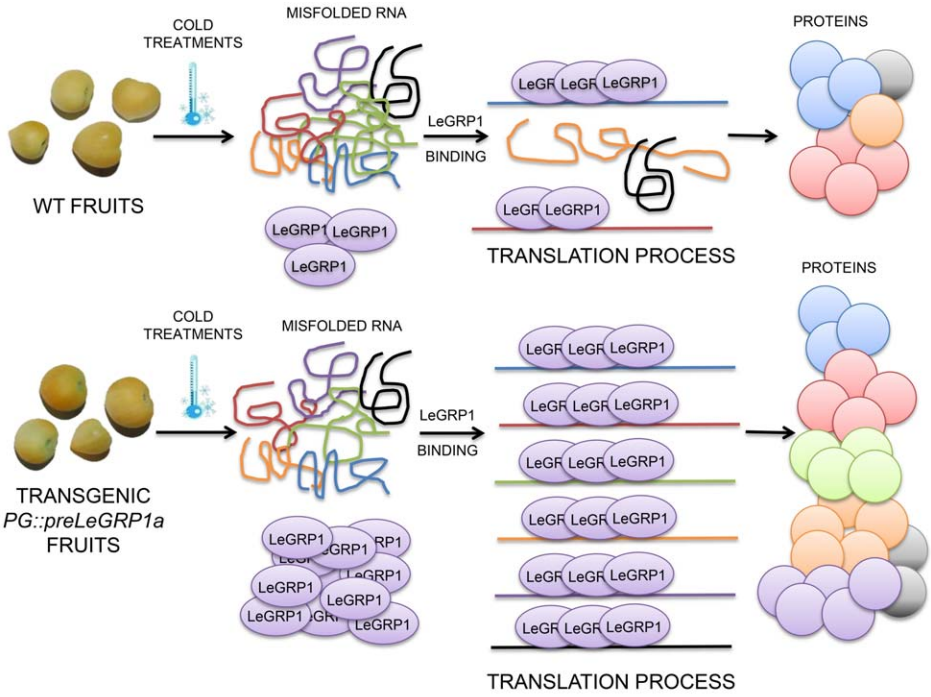

Fig. 5. A proposed model of LeGRP1-RNA complexes during cold treatments. When cold treatment is applied to fruits, processes such as protein synthesis, translation, and ripening are arrested. In this model, the increased LeGRP1 content in *PG::preLeGRP1a* fruits get importance in the structural mRNA rearrangements and/or stabilization of miss-folded RNA allowing protein translation.

In plants, cold shock domain (CSD) proteins play essential roles in freezing tolerance, and regulate embryo development, flowering time, and fruit development (Sasaki and Imai 2012). The fact that transgenic fruits had higher protein content than WT fruits after 7 d of cold treatment, but not when comparing non-treated fruits, reinforced the notion that LeGRP1 function as RNA chaperone in fruit during cold. We demonstrated in our previous work (Müller *et al*. 2014) that LeGRP1 harbours *in vivo* RNA chaperone activity (*in vivo* transcription antitermination abilities assayed in *E. coli* RL211 system) and so contributes to cold tolerance enhancement (by complementing *E. coli* BX04 mutant, which is highly sensitive to cold stress). Thus, it is likely that LeGRP1a facilitates translation by inducing structural rearrange-ment of mRNAs, which is specially

required for efficient translation under low temperatures. However, we cannot rule out that LeGRP1 could be stabilizing RNAs in a non-specific manner during cold, as it is demonstrated for other GR-RBPs in plants (Kim *et al*. 2008, Kwak *et al*. 2011, 2016, Khan *et al*. 2014). Overall, we postulated that, during ripening at ambient temperature, the overexpression of LeGRP1 in fruits did not lead to an increment in total protein content, since RNA is correctly folded and translation continues at normal rate (Fig. 5). However, under cold treatment, the increased LeGRP1 content in transgenic fruits induced structural rearrangements of mRNAs and/or stabilized miss-folded RNA allowing protein translation (Fig. 5). In addition, the formation of high molecular complexes of LeGRP1 with RNAs could be a plausible possibility, enabling RNA metabolism, as it was revealed for its homologue in *Nicotiana tabacum*, NtGR-RBP1, where intra-molecular interaction between the RNA-domain and the GR domain, as well as inter-molecular interaction between GR domains were demonstrated (Khan *et al*. 2014).

It is interesting to note that some amino acids like Gly and Lys, were more abundant in transgenic fruits than in WT fruits after cold storage. These amino acids are precursors of polyamines, alkaloids, and compatible solutes (Pratelli and Pilot 2014). Gly is a precursor of glycine-betaine, an osmoprotectant reported in tomato and other plants under stress conditions (Sakamoto and Murata 2002, Park *et al.* 2007). Probably, the over-expression of LeGRP1a could stabilize enzymes involved in the biosynthesis of these amino acids, whose transcripts may be particularly sensitive to cold. Future experiments are required to address these issues.

In conclusion, the data presented in this work revealed that the overexpression of *LeGRP1a* in tomato fruit led to a great induction of the other *LeGRP1* family members and so positive regulation among the *Legrp1* genes. Although *LeGRP1a* transcription and LeGRP1 protein content were increased, circadian profiles of LeGRP1 proteins were similar when comparing WT and transgenic fruits. Moreover, total protein content in chilled transgenic fruits was higher than in chilled WT fruits. Considering that *PG::preLeGRP1a* fruits do not exhibit detrimental changes in the organoleptic parameters neither under ripening at ambient conditions nor under cold storage, the overexpression of *LeGRP1a* in fruits emerge as a promising biotechnological tool to improve nutritional quality of tomatoes, by increasing higher total protein content and the content of precursors of some osmoprotectants.

References

Ambrosone, A., Batelli, G., Nurcato, R., Aurilia, V., Punzo, P., Bangarusamy, D. K., Ruberti, I., Sassi, M., Leone A., Costa, A., Grillo, S.: The *Arabidopsis* RNA-binding protein AtRGGA regulates tolerance to salt and drought stress. - Plant Physiol. **168**: 292-306, 2015.

An, G., Ebert, P.R., Mitra, A. Ha, S.B.: Binary Vectors in Plant Molecular Biology. - Kluwer Academic Publishers, Dordrect 1988.

Ayarpadikannan, S., Chung, E.S., So, H.A., Kim, K.M., Schraufnagle, K.R., Lee, J.H.: Overexpression of *SaRBP1* enhances tolerance of *Arabidopsis* to salt stress. - Plant Cell Tissue Organ Cult. **118**: 327-338, 2014.

Borsani, J., Budde, C.O., Porrini, L., Lauxmann, M.A., Lombardo, V.A., Murray, R., Andreo, C.S., Drincovich, M.F., Lara, M.V.: Carbon metabolism of peach fruit after harvest: changes in enzymes involved in organic acid and sugar level modifications. - J. exp. Bot **60**: 1823-1837, 2009.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal Biochem. **72**: 248-254, 1976.

Burnette, W.N.: "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. - Anal Biochem. **112**: 195-203, 1981.

Cook, K.B., Kazan, H., Suberi, K., Morris, Q., Hughes, T.R.: RBPDB: a database of RNA-binding specificities. - Nucl. Acids Res. **39**: 301-308, 2011.

Dhar, P., Tayade, A.B., Kumar, J., Chaurasia, O.P., Srivastava, R.B., Singh, S.B.: Nutritional profile of phytococktail from trans-Himalayan plants. - PLoS One **8**: e83008, 2013.

Fahnenstich, H., Saigo, M., Niessen, M., Zanor, M.I., Andreo, C.S., Fernie, A., Drincovich, M.F., Flügge, U.I., Maurino, V.G.: Alteration of organic acid metabolism in *Arabidopsis* overexpressing the maize C_4 NADP-malic enzyme causes accelerated senescence during extended darkness. - Plant Physiol. **145**: 640-652, 2007.

Fernandez, A.I., Viron, N., Alhagdow, M., Karimi, M., Jones, M., Amsellem, Z., Sicard, A., Czerednik, A., Angenent, G., Grierson, D., May, S., Seymour, G., Eshed, Y., Lemaire-Chamley, M., Rothan, C., Hilson, P.: Flexible tools for gene expression and silencing in tomato. - Plant Physiol. **151**: 1729-1740, 2009.

Jackman, R.L., Yada, R.Y., Marangoni, A., Parkinz, K.L., Stanley, D.W.: Chilling injury. A review of quality aspects. - J. Food Qual. **11**: 253-278, 1988.

Jung, H.J., Park, S.J., Kang, H.: Regulation of RNA metabolism in plant development and stress responses. - J. Plant Biol. **56**: 123-129, 2013.

Kang, H., Park, S.J., Kwak, K.J.: Plant RNA chaperones in stress response. - Trends Plant Sci. **18**: 100-106, 2013.

Khan, F., Daniëls, M.A., Folkers, G.E., Boelens, R., Saqlan Naqvi, S.M., Van Ingen, H.: Structural basis of nucleic acid binding by *Nicotiana tabacum* glycine-rich RNA-binding protein: implications for its RNA chaperone function. - Nucl. Acids Res. **42**: 8705-8718, 2014.

Kim, J.S., Jung, H.J., Lee, H.J., Kim, K.A., Goh, C.H., Woo, Y., Oh, S.H., Han, Y.S., Kang, H.: Glycine-rich RNA-binding protein7 affects abiotic stress responses by regulating stomata opening and closing in *Arabidopsis thaliana*. - Plant J. **55**: 455-466, 2008.

Kim, J.S., Park, S.J., Kwak, K.J., Kim, Y.O., Kim, J.Y., Song, J., Jang, B., Jung, C.-H., Kang, H.: Cold shock domain proteins and glycine-rich RNA-binding proteins from

Arabidopsis thaliana can promote the cold adaptation process in *E. coli*. - Nucl. Acids Res. **35**, 506-516, 2007b.

Kim, J.Y., Kim, W.Y., Kwak, K.J., Oh, S.H., Han, Y.S., Kang, H.: Glycine-rich RNA-binding proteins are functionally conserved in *Arabidopsis thaliana* and *Oryza sativa* during cold adaptation process. - J. exp. Bot. **61**: 2317-2325, 2010.

Kim, J.Y., Park, S.J., Jang, B., Jung, C.-H., Ahn, S.J., Goh, C.-H., Cho, K., Han, O., Kang, H.: Functional characterrization of a glycine-rich RNA-binding protein 2 in *Arabidopsis thaliana* under abiotic stress conditions. - Plant J. **50**: 439-451, 2007a.

Kim, M.K., Jung, H.J., Kim, D.H., Kang, H.: Characterization of glycine-rich RNA-binding proteins in *Brassica napus* under stress conditions. - Physiol. Plant **146**: 297-307, 2012.

Kwak, K.J., Kim, H.-S., Jang, H.Y., Kang, H., Ahn, S.-J.: Diverse roles of glycine-rich RNA-binding protein 7 in the response of camelina (*Camelina sativa*) to abiotic stress. - Acta Physiol. Plant. **38**: 129, 2016.

Kwak, K.J., Park, S.J., Han, J.H., Kim, M.K., Oh, S.H., Han, Y.S., Kang, H.: Structural determinants crucial to the RNA chaperone activity of glycine-rich RNA-binding proteins 4 and 7 in *Arabidopsis thaliana* during the cold adaptation process. - J. exp. Bot. **62**: 4003-4011, 2011.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. - Nature **227**: 680-685, 1970.

Lana, M.M., Tijskens, L.M.M., Van Kooten, O.: Effects of storage temperature and fruit ripening on firmness of fresh cut tomatoes. - Postharvest Biol. Technol. **35**: 87-89, 2005.

Lau, J.M., Cooper, N.G., Robinson, D.L., Korban, S.S.: Sequence and *in silico* characterization of the tomato polygalacturonase (PG) promoter and terminator regions. - Plant mol. Biol. Rep. **27**: 250-256, 2009.

López Camelo, A.F.: Storage. - In: Manual for thePreparation and Sale of Fruits and Vegetables from Field to Market. Pp. 47-74. FAO, Rome 2004.

Lorković, Z.J.: Role of plant RNA-binding proteins in development. stress response and genome organization. - Trends Plant Sci. **14**: 229-236, 2009.

Müller, G.L., Budde, C.O., Lauxmann, M.A., Triassi, A., Andreo, C.S., Drincovich, M.F., Lara, M.V.: Expression profile of transcripts encoding cell wall remodelling proteins in tomato fruit cv. Micro-Tom subjected to 15 °C storage. - Funct. Plant Biol. **40**: 449-458, 2013.

Müller, G.L., Triassi, A., Alvarez, C., Falcone Ferreyra, M.L., Andreo, C.S., Lara, M.V., Drincovich, M.F.: Circadian oscillation and development-dependent expression of glycine-rich RNA binding proteins in tomato fruits. - Funct. Plant Biol. **41**: 411-423, 2014.

Nagata, M., Yamashita, I.: Simple method for simultaneous determination of chlorophyll and carotenoids in tomato fruit. - J. jap. Soc Food Sci **39**: 925-928, 1992.

Park, E.J., Jeknić, Z., Pino, M.T., Murata, N., Chen, T.H.: Glycinebetaine accumulation is more effective in chloroplasts than in the cytosol for protecting transgenic tomato plants against abiotic stress. - Plant Cell Environ. **8**: 994-1005, 2007.

Phadtare, S.: Unwinding activity of cold shock proteins and RNA metabolism. - RNA Biol. **8**: 394-397, 2011.

Pratelli, R. Pilot, G.: Regulation of amino acid metabolic enzymes and transporters in plants. - J. exp. Bot. **65**: 5535-5556, 2014.

Sachetto-Martins, G., Franco, L.O., De Oliveira, D.E.: Plant glycine-rich proteins: a family or just proteins with a common motif? - Biochim. biophys. Acta **1492**: 1-14, 2000.

Sahi, C., Agarwal, M., Singh, A., Grover, A.: Molecular characterization of a novel isoform of rice (*Oryza sativa* L.) glycine rich-RNA binding protein and evidence for its involvement in high temperature stress response. - Plant Sci. **173**: 144-155, 2007.

Sakamoto, A., Murata, N.: The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. - Plant Cell Environ. **25**: 163-171, 2002.

Sasaki, K., Imai, R.: Pleiotropic roles of cold shock domain proteins in plants. - Front. Plant Sci. **2**: 116, 2012.

Schöning, J.C., Streitner, C., Meyer, I.M., Gao, Y., Staiger, D.: Reciprocal regulation of glycine-rich RNA-binding proteins *via* an interlocked feedback loop coupling alternative splicing to nonsense-mediated decay in *Arabidopsis* reciprocal regulation of glycine-rich RNA-binding proteins *via* an interlocked feedback loop. - Nucl. Acids Res. **36**: 6977-6987, 2008.

Schöning, J.C., Streitner, C., Page, D.R., Hennig, S., Uchida, K., Wolf, E., Furuya, M., Staiger, D.: Autoregulation of the circadian slave oscillator component AtGRP7 and regulation of its targetstargets is impaired by a single RNA recognition motif point mutation. - Plant J. **5**: 1119-1130, 2007.

Staiger, D., Zecca, L., Wieczorek Kirk, D.A., Apel, K., Eckstein, L.: The circadian clock regulated RNA-binding protein AtGRP7 autoregulates its expression by influencing alternative splicing of its own pre-mRNA. - Plant J. **33**: 361-371, 2003.

Tan, Y., Qin, Y., Li, Y., Li, M., Ma, F.: Overexpression of *MpGR-RBP1*, a glycine-rich RNA-binding protein gene from *Malus prunifolia* (Willd.) Borkh, confers salt stress tolerance and protects against oxidative stress in *Arabidopsis*. - Plant Cell Tissue Organ Cult. **119**: 635-646, 2014.

Zhang, B., Tieman, D.M., Jiao, C., Xu, Y., Chen, K., Fe, Z., Giovannoni, J.J., Klee, H.J.: Chilling-induced tomato flavor loss is associated with altered volatile synthesis and transient changes in DNA methylation. - Proc. nat. Acad. Sci. USA **113**: 12580-12585, 2016.