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Circadian oscillation and development-dependent expression of glycine-rich RNA binding proteins in tomato fruits

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Abstract. Glycine-rich RNA-binding proteins (GRPs) are involved in the modulation of the post-transcriptional processing of transcripts and participate as an output signal of the circadian clock. However, neither GRPs nor the circadian rhythmic have been studied in detail in fleshy fruits as yet. In the present work, the *GRP1* gene family was analysed in Micro-Tom tomato (*Solanum lycopersicum* L.) fruit. Three highly homologous *LeGRP1* genes (*LeGRP1a–c*) were identified. For each gene, three products were found, corresponding to the unspliced precursor mRNA (pre-mRNA), the mature mRNA and the alternatively spliced mRNA (*preLeGRP1a–c*, *mLeGRP1a–c* and *asLeGRP1a–c*, respectively). Tomato GRPs (LeGRPs) show the classic RNA recognition motif and glycine-rich region, and were found in the nucleus and in the cytosol of tomato fruit. By using different *Escherichia coli* mutants, it was found that LeGRP1s contained *in vivo* RNA-melting abilities and were able to complement the cold-sensitive phenotype of BX04 cells. Particular circadian profiles of expression, dependent on the fruits' developmental stage, were found for each *LeGRP1* form. During ripening off the vine of fruits harvested at the mature green stage, the levels of all *LeGRP1a–c* forms drastically increased; however, incubation at 4°C prevented such increases. Analysis of the expression of all *LeGRP1a–c* forms suggests a positive regulation of expression in tomato fruit. Overall, the results obtained in this work reveal a complex pattern of expression of *GRPs* in tomato fruit, suggesting they might be involved in post-transcriptional modulation of circadian processes of this fleshy fruit.

Additional keywords: alternative spliced products, circadian rhythm, post-transcriptional modulation, *Solanum lycopersicum.*

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Introduction

Glycine-rich RNA-binding proteins (GRPs) are widely distributed in organisms ranging from cyanobacteria to animals (Sachetto-Martins *et al*. 2000). These proteins contain two distinct domains: the *N*-terminal RNA-binding domain (also known as the RNA recognition motif (RRM) or the ribonucleoprotein domain (RNP)) and the glycine-rich *C*-terminal domain, which might be involved in protein–protein interactions. In plants, GRPs have been involved in many different processes, such as development, floral transition and flower development, genome organisation, and stress responses to cold, wound, salinity, flooding and pathogen infection (Sachetto-Martins *et al*. 2000; Staiger *et al*. 2003; Lorković 2009; Kim *et al*. 2012). They participate in the post-transcriptional RNA metabolism in both the cytoplasm and the nucleus (Ziemienowicz *et al*. 2003; Fusaro *et al*. 2007). The level of the transcripts encoding some GRPs shows oscillations regulated by the circadian clock (Carpenter *et al*. 1994; Heintzen *et al*. 1994; Heintzen *et al*. 1997; Staiger *et al*. 2003); furthermore, GRPs were suggested as part of the output signal of this clock (Staiger and Green 2011).

Within plants, GRPs have been characterised in *Arabidopsis thaliana* (L.) Heynh. (Kim *et al*. 2010), rice (*Oryza sativa* L.) (Kim *et al*. 2010), maize (*Zea mays* L.) (Gómez *et al*. 1988), *Malus prunifolia* (Willd.) Borkh (Wang *et al*. 2011), *Brassica napus* L. (Kim *et al*. 2012) and *Nicotiana tabacum* L. (Lee *et al*. 2009). In *A. thaliana*, eight GRP family members (AtGRP1–8) have been identified, with AtGRP2, -4 and -7 having an impact on seed germination and stress responses (Kwak *et al*. 2005; Kim *et al*. 2007*a*, 2007*b*, 2008, 2010). AtGRP7 has RNA chaperone activity and confers cold and freezing tolerance (Kim *et al*. 2008, 2010). Remarkably, AtGRP7 binds to its precursor mRNA (pre-mRNA) and promotes the formation of an alternatively spliced transcript, retaining an intron including a premature termination codon (Staiger *et al*. 2003; Schöning *et al*. 2007, 2008). Thus, this alternatively spliced *AtGRP7* is short-lived and results degraded via the nonsense-mediated decay pathway (Schöning *et al*. 2008). Moreover, AtGRP7 also regulates the alternative splicing of *AtGRP8* (Schöning *et al*. 2008). Alternative spliced transcripts of *GRP* have also been described in other plant species (Hirose *et al*. 1993). Despite increasing knowledge of

the diverse types of GRPs found in plants, their biological function and regulation of expression are not completely understood.

Fruit proteomic studies have identified several GRPs associated with peach (*Prunus persica* (L.) Stokes) ripening and response to stressing temperatures during storage after harvest (Borsani *et al*. 2009; Nilo *et al*. 2010) and with tomato (*Solanum lycopersicum* L.) response to the incubation at nonchilling low temperatures (Vega-García *et al*. 2010). Besides these proteomic studies, no previous studies have evaluated the mechanisms of expression of the different *GRPs* during development and after harvest, and the roles that these proteins may have in fleshy fruits. Considering that GRPs have been related to chilling tolerance in *A. thaliana* (Kim *et al*. 2008), the study of these proteins in chilling sensitive fruits is of great relevance (Hobson 1987; Müller *et al*. 2013). In the present work, we determined the circadian oscillations in the expression of three *GRPs* at different developmental stages in dwarf tomato (*S. lycopersicum* cv. Micro-Tom) fruits and in fruits stored under different temperature conditions. Tomato GRPs (LeGRP1s) were found in the nucleus and in the cytoplasm of the fruit, and their *in vivo* functionality was assessed using different *Escherichia coli* mutants. Overall, the results obtained indicate particular patterns of expression for each *GRP*, which were dependent on the developmental stage. Taken as a whole, these results reveal a complex pattern of expression of different *GRPs* in tomato fruit and point out these proteins as possible participants of post-transcriptional control in this fruit, possibly linked to circadian processes.

Materials and methods

Plant material and storage conditions

Seeds of *Solanum lycopersicum* cv. Micro-Tom (Scott and Harbaugh 1989) were surface-sterilised for 10 min in 5% (v/v) sodium hypochlorite containing 0.05% (w/v) Tween 20, then thoroughly rinsed with tap water for another 3 min and finally germinated in 500-mL pots with sterile soil. Plants were grown in chambers under 16 h of fluorescent light at a PPFD of 200 µmol m^{-2} s^{-1} and 8 h darkness with day : night temperatures of 22°C : 17°C. Flowers were tagged after pollination and fruit were allowed to develop to different stages according to the number of days after anthesis (DAA) and harvested for analysis or used for the post-harvest treatments.

Tomatoes were harvested at the immature green (IG, 16 DAA), mature green (MG, 36 DAA) and red ripe (RR, 45 DAA) stages. For time-course analysis, IG, MG and RR fruits were harvested from the vine grown in 16-h : 8-h light–dark cycles at 4-h intervals (Zeitgeber time (ZT) 0, ZT 4, ZT 8, ZT 12, ZT 16 and ZT 20). Also, tomato plants with IG, MG or RR fruits grown under a 16-h light : 8-h dark photoperiod were transferred to continuous light for a 24-h period and fruits were harvested at 4-hourly intervals (ZT 0, ZT 4, ZT 8, ZT 12, ZT 16 and ZT 20). Time-course analysis was also performed on fruits harvested from the vine at the IG, MG or RR stages. Harvested fruits were kept in the chamber continuing the same light–dark period as the whole plant for 24 h and sampled at ZT 0, ZT 4, ZT 8, ZT 12, ZT 16 and ZT 20 of the following light–dark cycle after separation from the plant. Time-course analyses were performed on fruits collected from different plants and during different days.

To analyse the changes in *GRP* expression during storage, fruits were harvested at the MG stage and stored at 4°C for 7 days (7D4), or at 20°C for 7 (7D20) or 9 days (9D20) in dark chambers with a relative humidity of 90–93%. Fruits stored at 4°C were transferred to 20°C for 2 days (7D4 + 2). Each treatment was repeated at least three times.

At each sampling time, fruits were cut into pieces; gel and seeds were removed and the remaining pericarp was frozen in liquid nitrogen and stored at −80°C for extraction of protein and RNA.

Protein extraction and quantitation

Total soluble protein from the different samples was extracted using a buffer containing 50 mM KH_2PO_4 (pH 7.0), 1 mM EDTA, 1 mM ascorbate, 20% (v/v) glycerol, 1 mM phenylmethylsulfonylfluoride and 33 µg mL^{-1} protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The samples were ground in a mortar using liquid nitrogen and centrifuged at 10 000*g* for 10 min at 4°C. The crude extract supernatant was diluted in 0.25 M Tris-HCl (pH 7.5), 2% (w/v) SDS, 0.5% (v/v) β-mercaptoethanol and 0.1% (v/v) bromophenol blue, and was boiled for 2 min for SDS-PAGE. Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) and BSA as standard. For visualising protein loading control before western blot analysis (Fig. S3, available as Supplementary Material to this paper) samples were loaded in 12 % SDS-PAGEs and gels were stained with Coomassie Blue (0.1% Coomassie Blue R250 in 10% acetic acid and 50% methanol).

LeGRP1a–His-Tag fusion protein

In order to obtain the LeGRP1a coding region, oligonucleotides primers (5′CC*ATGG*CCGCCGAAGTTGAGTA3′ and 5′CTCGAGATAACGGTCACCACC3′) covering the translation start codon (italics) and comprising engineered NcoI and XhoI sites (underlined) were used to perform PCRs using cDNA of IG fruits. The PCR product was cloned into *pGEMT-Easy* (Promega, Madison, WI, USA). Plasmid was digested with NcoI-XhoI and the insert was subcloned into *pET 28b+* (Novagen, Darmstadt, Germany). Recombinant LeGRP1a–His-Tag was obtained by induction of 200 mL of *E. coli* BL21 DE3 culture (Optical Density (OD) 0.9–1) with 100 mM of isopropyl-D-thiogalactopyranoside (IPTG) for 6 h at 30°C. Recombinant LeGRP1a–His-Tag protein was purified with a Ni-NTA column according to the manufacturer specifications (Promega). Polyclonal antibodies against recombinant LeGRP1a were obtained by immunising rabbits with two subcutaneous injections of 150 µg purified protein with Freund's incomplete and complete adjuvants (1 : 1), respectively; each performed at different intervals from the extraction of the preimmune serum. The antibodies obtained were further purified from the crude antiserum using recombinant LeGRP1a–His-Tag protein (Plaxton 1989).

SDS-PAGE and western blot analysis

SDS-PAGE was carried out using 15% (w/v) polyacrylamide gels according to Laemmli (1970). Gels were stained with Coomassie (0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol) during 1 h and unstained with destaining solution (10% acetic acid, 50% methanol). Bound antibodies were located by linking to alkaline phosphatase-conjugated goat antirabbit immunoglobin according to the manufacturer's instructions (Bio-Rad). Purified antibodies raised against LeGRP1a (1 : 1000) were used. In addition, antibodies raised against *Sinapis alba* L. GRP (SaGRP, 1 : 2500) that cross-react with AtGRP7 and also display a weaker reaction against AtGRP8 (kindly provided by Prof. Dr Dorothee Staiger) were also used in the analysis.

RNA isolation and reverse transcription–PCR

Total RNA from tomato fruits was isolated from 0.04–0.10 g of tomato pericarp using the Trizol method, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), with the following modifications: after the addition of isopropanol, the pellet obtained was resuspended in 200 µL of distilled water treated with diethylpyrocarbonate and RNA was precipitated by addition of 2.5 volumes of absolute ethanol for 2 h at −20°C. After three washes with 80% (v/v) ethanol, the pellet was dried and resuspended in 40 µL of distilled water that was free of RNAses for 20 min at 55°C. The integrity of the RNA was verified by agarose electrophoresis. The quantity and the purity of the RNA were determined spectrophotometrically. First-strand cDNA was synthesised with Moloney murine leukemia virus (MoMLV)-reverse transcriptase following the manufacturer's instructions (Promega) and using 2 µg of RNA and oligo(dT).

mLeGRP1a, asLeGRP1b *and* asLeGRP1c *isolation and cloning*

mLeGRP1a was isolated as described above using primers for cloning in *pET28b+* (Novagen). *asLeGRP1b* and *asLeGRP1c* were isolated from the cDNA of IG fruits. Oligonucleotides primers were 5′-ATTCCAATTCCCCGAAACGATCATC-3′ (Forward) and 5′-CTAATTCCTCCAGTTCCCAT-3′ (Reverse) for *asLeGRP1b*, and as 5′-ATTCCATTCCTTTATACGATT-3′ (Forward) and as 5′-CCTAATTCCTCCAGCTTCCT-3′ (Reverse) for *asLegrp1c*. PCR products were purified and the corresponding sequences were analysed.

Quantitative real-time PCR

Relative expression was determined by performing quantitative real-time PCR (qPCR) in an iCycler iQ detection system and the Optical System software ver. 3.0a (Bio-Rad, Hercules, CA, USA), using the intercalation dye SYBRGreen I (Invitrogen) as a fluorescent reporter, with 2.5 mM $MgCl_2$, 0.5 µM of each primer and 0.04 U µL^{-1} GoTaq (Promega). PCR primers were designed based on tomato cDNA sequences published in GenBank and *S. lycopersicum* EST databases (TIGR Plant Transcript Assemblies (http://plantta.tigr.org, Childs *et al*. 2007); Sol Genomics Network (http://solgenomics.net/)), with the aid of the web-based program Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) in a way to produce amplicons of 150–300 bp in size (see Table S1, available as Supplementary Material to this paper). A 10-fold dilution of cDNA obtained as described above was used as a template. PCR controls were performed in the absence of added reverse transcriptase to ensure that RNA samples were free of DNA contamination. Cycling parameters were as follows: initial denaturation at 94°C for 2 min, 40 cycles of 96°C for 10 s and 58°C for 15 s, 72°C for 1 min and 72°C for 10 min. The SYBRGreen I fluorescence of the double-strand amplified products was measured at 78°C. Melting curves for each PCR reaction were determined by measuring the decrease of fluorescence with increasing temperature (from 65°C to 98°C). The specificity of the PCR reactions was confirmed by melting curve analysis using the software MxPRO QPCR Software (Stratagene, CA, USA) as well as by agarose gel electrophoresis of the products. Furthermore, PCR products were sequenced by Macrogen Inc. (Seoul, Korea). Amplification efficiency for each gene was determined using the relative standard curve method (Čikoš *et al*. 2007). Relative gene expression was calculated using the comparative $E^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) and *Elongation Factor 1 α* (*LeEF1α*) as the reference gene. Each cDNA sample was run in technical triplicate and repeated in at least three independent sets of samples.

Transient expression and subcellular localisation analysis

Tomato fruits at the MG stage (37 days after anthesis) were used for transient expression experiments. Agroinjection with *p35S*::*preLegrp1a-c*:*Green Fluorescent Protein (GFP)* and controls (*p35S*::*GFP* and *p35S*) were performed 4 days before the MG stage in attached fruits following previously described protocols (Orzaez *et al*. 2006). After 7 days, the fruits were hand-cut into thin slices and then incubated for 15 min in 2 µg mL^{-1} of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) in PBS buffer (10 mM sodium phosphate, 130 mM NaCl; pH 7.2). After three washes with PBS, the slices were mounted in 50% glycerol. The fluorescence of GFP and DAPI was visualised by confocal laser scanning microscopy (CLSM) (Nikon C1, Tokyo, Japan) with a 40× objective. GFP and DAPI were excited using an argon laser at 488 nm and a UV laser at 395 nm, respectively. GFP emission was collected between 515 nm and 530 nm to avoid crosstalk with chloroplast autofluorescence. More than 100 nuclei were analysed (20 images) from different fruits. The experiments were repeated at least three times.

Transcription antitermination and complementation assays in E. coli

An *in vivo* transcription antitermination assay in *E. coli* RL211 (obtained from Dr R. Landick, Landick *et al*. 1990) was conducted essentially as described by Kim *et al*. (2012). Briefly, *mLeGRP1a–c* cDNA and *CspA* coding sequences were cloned into the SacI/*Hin*dIII site of the *pBluscript SK(-)* vector (*pBS*) and the vectors were introduced into *E. coli* RL211. Transformed bacteria were grown in liquid Luria-Bertani (LB) medium with ampicillin and spotted on LB-ampicillin plates with or without chloramphenicol at 37°C. Cold shock assays were carried out in BX04 cells (kindly provided by Dr Masayori Inouye and Dr Sangita Phadtare). BX04 mutant cells (Xia *et al*. 2001) lack four cold shock proteins and are highly sensitive to cold stress. BX04 cells were transformed with each construct and

cells containing either *pBS–LeGRP1a–c*, *pBS–CspA* (positive control) or *pBS* (negative control) were grown in LB medium containing ampicillin and kanamycin. Serial-diluted cultures were spotted on LB medium with or without 0.1 mM IPTG and incubated at 37°C overnight or at 18°C over 5 days. The growth of the cells was inspected daily.

Promoter sequence analysis

The genomic 1 or 3 kb upstream of the ATG start codon regions of *LeGRP1a–c* were used for analysis with the PLACE signal scan program (http://www.dna.affrc.go.jp/htdocs/PLACE/; Higo *et al*. 1999). These regions included the 5′ UTR and the proximal promoter.

Statistical analysis

Data from the quantitative real time experiments were tested using one-way ANOVA. Minimum s.d. was calculated by the Bonferroni, Holm–Sidak, Dunett and Duncan tests ($\alpha = 0.05$) using the Sigma Stat package (R Systat Software Inc. (SSI), San Jose, CA, USA). Spearman rank order correlation analyses were done with the Sigma Stat package.

Accession numbers

Sequence data from this article can be found in the European Molecular Biology Laboratory (EMBL) and GenBank data libraries under accession numbers JQ613215, JQ613216 and JQ613217 (Table 1).

Results

Analysis of GRP transcripts from Solanum lycopersicum

As a first step towards the analysis of GRPs in fruits of *S. lycopersicum*, a BLAST search of the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Sol Genomics Network (http://solgenomics.net/tools/blast/index.pl) databases using *A. thaliana AtGRP7* and *-8* as input was performed. Six different transcript sequences were identified (*preLeGRP1a*, *asLeGRP1a*, *preLeGRP1b*, *mLeGRP1b*, *preLeGRP1c* and *mLeGRP1c*; Table 1). Sequence homology analysis among these transcripts indicates that they fall into three subgroups (*LeGRP1a–c*) with two transcripts each. The comparison of these sequences with those of *A. thaliana* and *S. alba GRPs* reveals that, among each group, the related sequences correspond to different spliced forms. Thus, within each group, the largest sequence corresponds to the unspliced pre-mRNA, called *preLeGRP1.* Introns located within the RNA binding domain of each GRP (RRM, Fig. 1) of 278 bp, 321 bp and 273 bp are retained in *preLeGRP1a–c*, respectively (Fig. 1). The others forms identified in the databases correspond to the mature mRNA, called *mLeGRP1b* and *mLeGRP1c*; an alternative spliced transcript of *LeGRP1a* (named *asLeGRP1a*), which retains

Table 1. Transcriptional glycine-rich RNA binding protein (GRP) forms in tomato

The locus name (Sol Genomics Network (SGN)) of the corresponding genes and accession numbers (National Center for Biotechnology Information (NCBI)) are indicated, as well as the length and particular features of each transcriptional form. Bold typeface indicates the sequences obtained in this work. UTR, untranslated region

Transcriptional form	SNG gene locus name	GenBank accession number	Length (bp)	Additional information
preLeGRP1a	Solyc10g051390	AK246918	992	39 bp 5′ UTR; 278-bp intron between 148 and 426 bp; 172 bp 3′ UTR
mLeGRP1a	Solyc10g051390	JQ613215	714	39 bp 5′ UTR and 172 bp 3′ UTR
asLeGRP1a	Solyc10g051390	AK323933	889	65 bp 5′ UTR; 151-bp intron between 176 and 327 bp; 172 bp 3′ UTR
preLeGRP1b	Solyc01g109660	AK320592	1140	57 bp 5′ UTR; 321-bp intron between 165 and 486 bp; 193 bp 3′ UTR
mLeGRP1b	Solyc01g109660	AK224744	785	44 bp 5′ UTR; 193 bp 3′ UTR
asLeGRP1b	Solyc01g109660	JQ613216	931	57 bp 5′ UTR; 112-bp intron between 165 and 277 bp; 193 bp 3′ UTR
preLeGRP1c	Solyc10g051380	AK324061	954	29 bp 5′ UTR; 273-bp intron between 140 and 413 bp; 150 bp 3′ UTR
mLeGRP1c	Solyc10g051380	AK323723	689	25 bp 5′ UTR and 150 bp 3′ UTR
asLeGRP1c	Solyc10g051380	JQ613217	824	29 bp 5′ UTR; 131-bp intron between 140 and 271 bp; 150 bp 3′ UTR

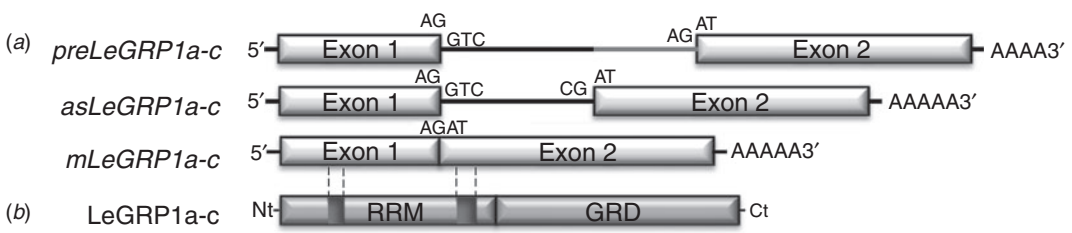

Fig. 1. Schematic representation of (*a*) precursor, alternative spliced and mature *LeGRP1a–c* RNAs and (*b*) LeGRP1a–c proteins in tomato. The boxes indicate the exons. The black and grey lines represent the two halves of the intron. The nucleotides homologous to the plant consensus splice site (Heintzen *et al*. 1994) are indicated. Schematic representation of the RNA recognition motif (RRM) and the glycine-rich domain (GRD) are shown in (*b*). Ribonucleoprotein domains 1 and -2 (RNP1 and RNP2, respectively) are illustrated by dark grey boxes.

151 bp of the 5′ first intron (Fig. 1, Table 1; Fig. S1*a*, *b*, available as Supplementary Material to this paper).

In the present work, three additional *LeGRP1 transcripts* were isolated from tomato fruit (Table 1), the sequences of which correspond to the mature transcript of *mLeGRP1a* (GenBank accession number JQ613215, Table 1) and the alternative spliced products of *LeGRP1b* and *LeGRP1c* (*asLeGRP1b* and *-c*, GenBank accession numbers JQ613216 and JQ613217, respectively, Table 1). Alignments of the *mLeGRP1a–c* and their respective pre-mRNAs show the presence of an intron in an identical location within eukaryotic genes that encode GRP proteins (Fig. S1*a*, *b*; Wang *et al*. 2011). Fig. 1*a* shows a schematic representation of *preLeGRP1a–c*, *asLeGRP1a–c* and *mLeGRP1a–c* transcripts, and Table 1 summarises the characteristics of each sequence, such as 5′ and 3′ UTRs, open reading frames and location of the introns. A BLAST search of the identified sequences to the tomato genome database (http://solgenomics.net/tools/blast/index.pl) indicates that the *LeGRP1a* and *LeGRP1c* genes are encoded in Chromosome 10 (Locus Solyc10g051390 and Solyc10g051380, respectively) and the *LeGRP1b* gene is encoded in Chromosome 1 (Solyc01g109660, Table 1).

Mature mRNA of *LeGRP1a*, *LeGRP1b* and *LeGRP1c* contain an open reading frame that predicts proteins of 164, 175 and 163 amino acid residues, with predicted molecular masses of 15.9 kDa, 17.3 kDa and 16.5 kDa, respectively. Importantly, each predicted protein contains all the consensus domains characteristic of the RNA binding protein superfamily, including an RRM with the two RNA binding sites (Ribonucleoprotein domain (RNP)1, RGFGFVTF; and RNP2, CFVGGL; Fig. 1*b* and Fig. S1*c*). Moreover, conserved amino acid residues interspersed throughout the RRM motif, as well as the typically glycine-rich *C*-terminal domain containing 2–5 glycine repeats bordered by arginine, tyrosine or both are found in the three predicted proteins (Fig. S1*c*; Stephen *et al*. 2003; Kumaki *et al*. 2004). Transmembrane and signal peptide predictions indicate that LeGRP1a–c might not be secreted proteins (TargetP, http://www.cbs.dtu.dk/services/TargetP/). Prediction analysis using the PSORT algorithm (http://psort.ims.u-tokyo.ac.jp) indicates that these proteins could be localised in the nucleus.

The amino acid identity among the three LeGRPs is between 75% and 76% (Table 2). Cluster analysis of related and characterised protein sequences revealed that LeGRP1a is highly similar to *Nicotiana sylvestris* Speg. et Comes NsGRP1a, -1b and -1c (75%, 90%, and 73%, respectively, Table 2) and similar to AtGRP7 and AtGRP8 from *A. thaliana* (77% and 80%, respectively, Table 2). Also, LeGRP1b and -1c show the highest identity with NsGRP1c (86% and 80%, respectively), and 72% and 67% with AtGRP8. Protein BLAST analysis also revealed high identities with GRPs from *O. sativa* (ABF98117), *Solanum tuberosum* L. (ABB87126), *Catharanthus roseus* L. (AF200323), *N. attenuata* T. (ABH07505), *Vitis vinifera* L. (CBI32594), *Populus trichocarpa* Torr. & A.Gray (ABK92779), *Z. mays* (ACN25391) and soybean (*Glycine max* (L.) Merr.) (AAD48471).

In vivo *transcription antitermination abilities and complementation assay of LeGRP1 in* E. coli

GRPs of *A. thaliana*, *B. napus* and rice are RNA chaperones and confer cold tolerance in prokaryotes (Kim *et al*. 2007*b*; Kwak *et al*. 2011; Kim *et al*. 2012). To determine whether LeGRP1s have RNA chaperone activity *in vivo*, the transcription antitermination assay system developed by Landick *et al*. (1990) was carried out. *E. coli* RL211 strain contains a chloramphenicol resistance gene downstream from the *trpL* terminator and functions as an efficient system by which the transcription anti-termination activity can be assessed (Kim *et al*. 2012). The coding sequences of *LeGRP1a–c*, as well as the *E. coli CspA* gene as a positive control, were inserted into a *pBS* vector. The *pBS–GRP1a–c* constructs were transformed into RL211 cells and the *in vivo* transcription antitermination activity was evaluated. RL211 cells expressing *CspA* or *LeGRP1a–c* grew on the growth media containing chloramphenicol, whereas the RL211 cells containing the *pBS* vector were not able to grow on these plates (Fig. 2*a*). These results show that LeGRP1a–c proteins are capable of melting the secondary structure of RNA *in vivo*.

To test the functional role of LeGRP1a–c in cold stress, we examined each LeGRP1 for its ability to complement defects in the growth of BX04 cells at low temperature. The vectors used in the antitermination assays were introduced to *E. coli* BX04 cells and the colony-forming abilities were examined on LB plates at 18°C with or without IPTG. When the BX04 cells containing each construct were incubated at 37°C, all cells grew well without any noticeable difference (Fig. 2*b*). In contrast, when the cells were subjected to cold shock at 18°C, the growth of BX04 expressing LeGRP1a–c was higher than that of the control cells containing the empty vector (Fig. 2*b*). In

Table 2. Sequence identity (%) of LeGRP1a–c with glycine-rich RNA binding proteins (GRPs) from other plants
Nicotiana sylvestris (*NsGRP1a-c*, BAA03741, BAA03742 and BAA03743, respectively; Hirose *et al*. 1993) and *Arabidopsis thaliana* GRPs (AtGRP7 and AtGRP8, NP850017 and NP849523; Carpenter *et al*. 1994) were used for sequence comparison.

	LeGRP1a	LeGRP1b	LeGRP1c	NsGRP1a	NsGRP1b	NsGRP1c	AtGRP7
AtGRP8	80	72	67	76	76	70	77
AtGRP7	77	70	67	76	80	69	
NsGRP1c	73	86	80	81	78		
NsGRP1b	90	85	77	81			
NsGRP1a	75	85	74				
LeGRP1c	75	76					
LeGRP1b	76						

accordance with this, the BX04 cells expressing LeGRP1a–c protein did not form filamentous cells at a low temperature (Fig. S2) as happened with the cells containing the empty *pBS* vector. These results demonstrate that *LeGRP1* genes suppress the cell division defect of BX04, enabling the protection of *E. coli* BX04 against cold shock stress.

LeGRP1a–c *expression levels during tomato fruit development*

The relative level of the different forms of *LeGRP1a–c* was investigated in *S. lycopersicum* cv. Micro-Tom fruit harvested at ZT 0 at three different stages of development: the IG, MG and the RR stages by qPCR. Specific primers were designed in order to quantify the different transcriptional forms; the PCR products amplified were corroborated by sequencing. When comparing the level of expression of the three different forms of each *LeGRP*, it is notable that at the IG stage, the expression of the three mature transcript forms (*mLeGRP1a*, *b* and *c*) is higher than their respective pre-mRNA and alternative spliced forms (Fig. 3*a*). Also, comparing the three different *LeGRP1* genes, *mLeGRP1b* displays the highest level of expression (Fig. 3*a*). It is also notable that during the transition from IG to RR, the expression of

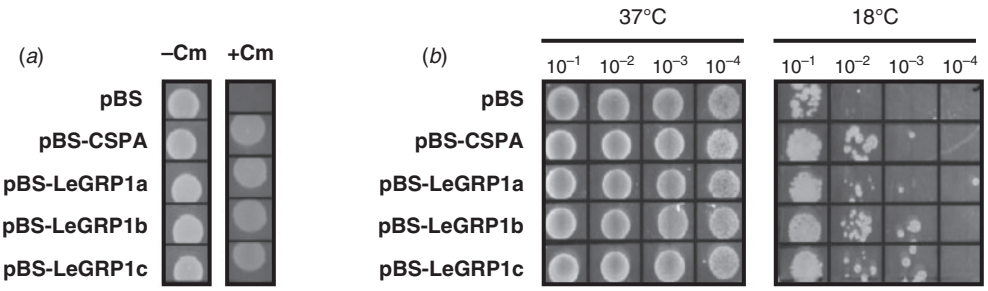

Fig. 2. *In vivo* transcription anti-termination abilities and complementation assay of LeGRP1 in *E. coli.* (*a*) Liquid cultures of RL211 cells (Optical Density $(OD)_{600nm} = 1$) containing *pBS–GRP1a*, *pBS–GRP1b*, *pBS–GRP1c* or *pBS–CspA* (positive controls), or the *pBS* vector (negative control) were spotted on Luria-Bertani (LB) agar with or without chloramphenicol (Cm), and the cells were grown overnight at 37°C. The image was obtained 1 day later. (*b*) Cultures of BX04 cells containing each construct ($OD_{600nm} = 1$) were diluted (10^{-1} to 10^{-4} dilution), spotted on LB agar plates and incubated at 18°C or 37°C; images were acquired 5 days or 1 day after incubation, respectively.

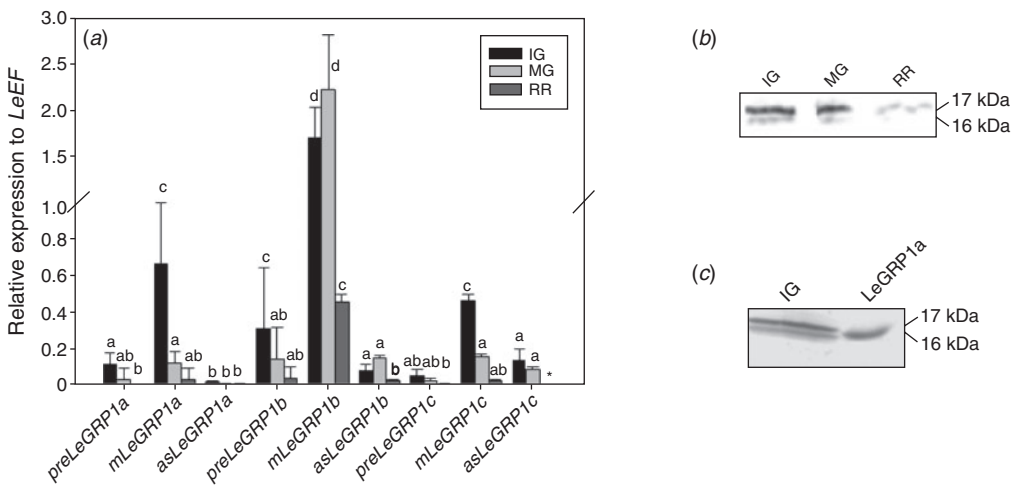

Fig. 3. Expression analysis of the different *LeGRP1a–c* forms and proteins in tomato cv. Micro-Tom fruits at different stages. Tomato cv. Micro-Tom fruits ripening on the vine were harvested at Zeitgeber time (ZT) 0 at the immature green (IG), mature green (MG) and red ripe (RR) stages. (*a*) *LeGRP1a–c* premature (pre), mature (m) and alternative spliced (as) mRNAs were quantified by reverse transcription real time PCR. The means of the results obtained, using three independent mRNAs as the template, are shown. The *y*-axis refers to the fold difference in a particular transcript level relative to the C_t values corresponding to *LeEF1*α; the s.d. is shown. Bars with the same letters are not significantly different ($P < 0.05$). Asterisks (*) indicate no amplification. (*b*, *c*) Western blot analysis of LeGRP1 revealed with antibodies raised against LeGRP1a. Thirty µg of pericarp protein was loaded in each lane in (*b*) and in the left lane of (*c*). Ten ng of recombinant LeGRP1a was loaded in the right lane of figure (*c*). Immunoreactive bands of 16 kDa and 17 kDa are shown.

practically all transcripts analysed decrease, irrespective of the type or form (Fig. 3*a*).

Additionally, antibodies against recombinant purified LeGRP1a were raised, purified and used to analyse immunoreactive LeGRP1 protein levels at each stage. Two immunoreactive bands of 17 and 16 kDa are detected in all the stages analysed (IG, MG and RR fruits, Fig. 3*b*). Considering the high degree of identity between the three LeGRPs, the antibodies against LeGRP1a may cross-react with LeGRPb, LeGRPc or both, explaining the presence of more than one band in crude extracts. Accordingly to the decrease in *LeGRP1* levels, the intensity of the two immunoreactive bands is also reduced in the transition from the IG to the RR stage (Fig. 3*b*). SDS-PAGEs showing loading control of crude protein extracts are shown in Fig. S3. The mobility of the lower band detected in fruit extracts corresponds to that of the recombinant LeGRP1a

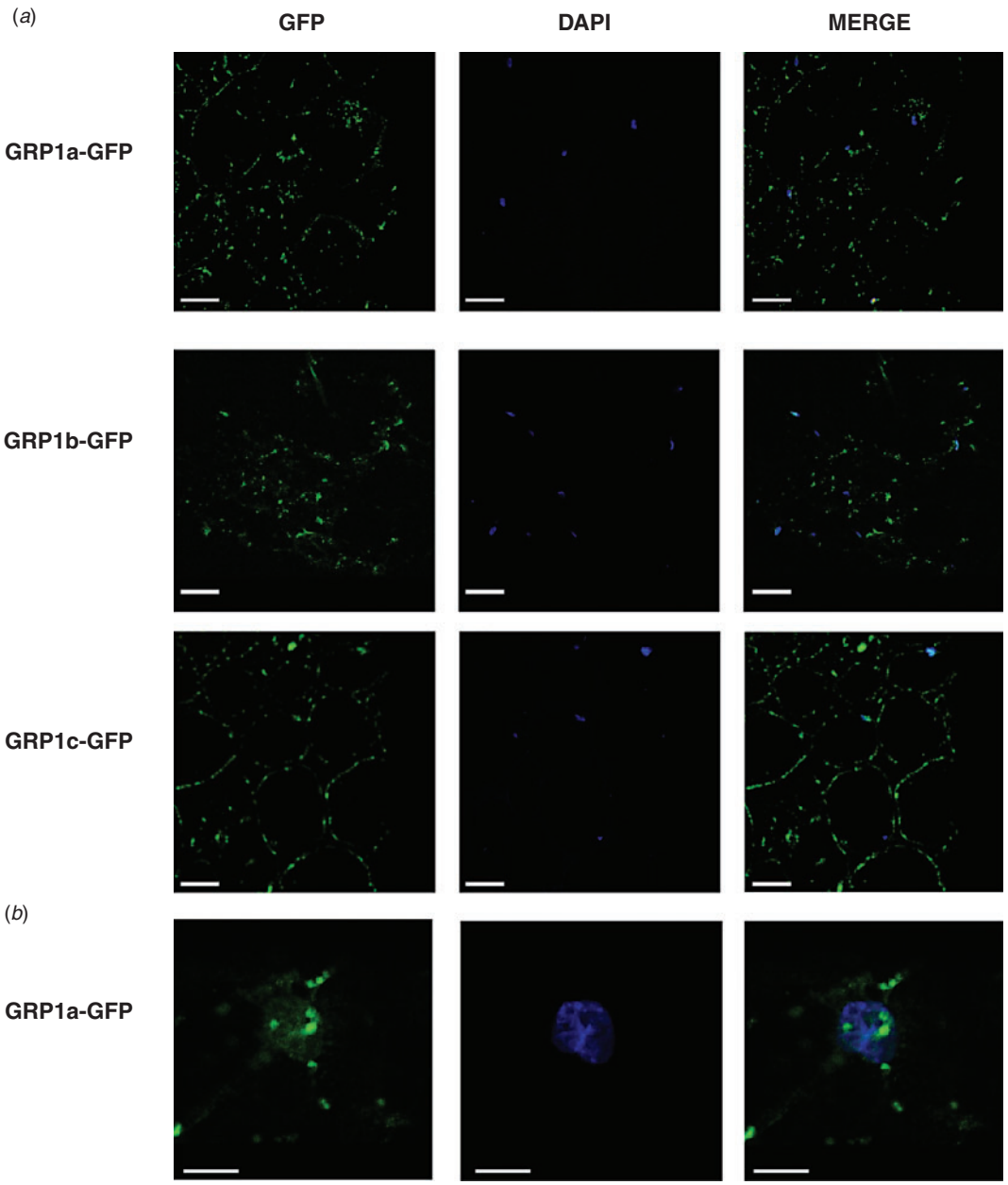

Fig. 4. Subcellular localisation of LeGRP1a–c in the cells of tomato fruits. (*a*) Confocal microscopy showing DAPI and GFP fluorescences in cells of mesocarp tissues transiently expressing *LeGRP1s–GFP* (7 days after infiltration). DAPI fluorescence indicates the position of the nuclei; GFP fluorescence indicates the localisation of LeGRP1s–GFP. Images were merged to show signal overlap. Bars: 50 µm. (*b*) Amplified scanning of a nucleus transiently expressing LeGRP1a–GFP, showing the formation of speckles. The formation of speckles in the nucleus was also observed for LeGRP1b–GFP and LeGRP1c–GFP (not shown). Bars: 10 µm.

(Fig. 3*c*). Immunoreactive bands with the same molecular mass (not shown) are also detected when using antibodies against *S. alba* GRP (Heintzen *et al*. 1994), although the reaction is weaker.

LeGRP1a–c proteins are found in the cytosol and in the nucleus

LeGRP1a to -c–GFP fusion proteins were examined by confocal microscopy of infiltrated *S. lycopersicum* cv. Micro-Tom fruits. All fusion proteins are detected in the cytosol and in the nuclei, which were stained with DAPI (Fig. 4). GFP protein is found in the cytosol in control plants infiltrated with *p35S*::*GFP* and no GFP signal is observed in uninfiltrated fruits (Fig. S4). Interestingly, the fusion protein formed speckles in the nucleus (Fig. 4*b*). Analysis of confocal optical sections confirmed that the speckles were distributed within the nucleus. Although speckles were observed in all of the nuclei, the number per nucleus varied. In most cases, one nucleus contained 5–10 speckles.

Time-course of transcript and protein LeGRP1 accumulation in S. lycopersicum *fruit*

The time-course profile over a 24-h period of the levels of the different forms of *LeGRP1a–c* and of LeGRP1 protein was analysed in fruits by qPCR and by western blot, respectively (Fig. 5). The results obtained indicate particular patterns of expression during the diurnal cycle for the three forms of each *LeGRP1* gene, patterns which also depend on the stage of development (Fig. 5). Regarding the mature forms, *mLeGRP1a* undergoes oscillations in IG, MG and RR fruits grown in

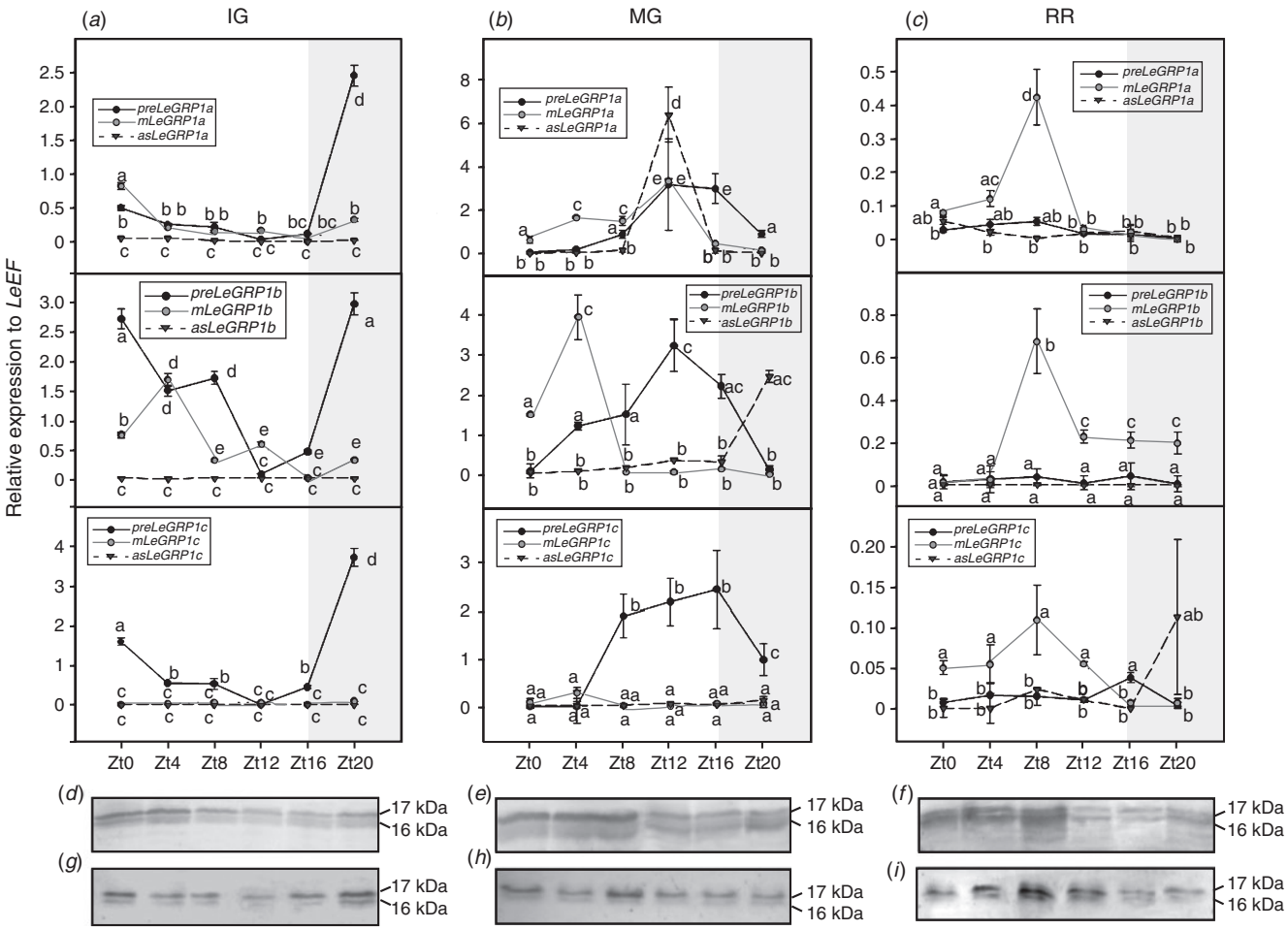

Fig. 5. *LeGRP1a–c* premature (pre), mature (m) and alternative spliced (as) mRNA and protein cycling in *S. lycopersicum* fruits. Fruits at the (*a*, *d*) immature green (IG), (*b*, *e*) mature green (MG) and (*c*, *f*) red ripe (RR) stages from plants under 16 : 8 light–dark cycle were harvested at 4-hourly time intervals. Light and dark periods are represented by white and grey areas, respectively; and the Zeitgeber time (ZT) is indicated in the graph. The expression analysis is shown by (*a–c*) quantitative real-time PCR of the different transcriptional forms and (*d–f*) western blot analysis. The means of the results obtained, using three independent mRNAs as template, are shown. The *y*-axis refers to the fold difference in a particular transcript level relative to the C_t values corresponding to *LeEF1*α; the s.d. is shown. For each curve, points with the same letters are not significantly different ($P < 0.05$). Western blots were conducted with antibodies against LeGRP1a and 30 µg of pericarp protein extracts were loaded. Immunoreactive bands of 16 kDa and 17 kDa are shown. Plants with (*g*) IG, (*h*) MG or (*i*) RR fruits were transferred to continuous light for a 24-h period and fruits were harvested at 4-hourly intervals. Western blot studies using 30 µg of pericarp protein are shown.

light–dark cycles with the highest levels (with an ~40-fold increase with respect to the minimum level) at ZT 0 in IG fruits, at ZT 12 in MG fruits and at ZT 8 in RR fruits (Fig. 5*a–c*). *mLeGRP1b* displays the highest levels 4 h after the onset of illumination in green stages (IG and MG) and at ZT 8 in RR fruits, with an ~80-fold increase with respect to the minimum level (Fig. 5*a–c*). In contrast, low and practically invariable levels of *mLeGRP1c* are detected in all three developmental stages (Fig. 5*a–c*).

Interestingly, *preLeGRP1a* to -*c* display similar pattern of time-course oscillation at each stage of development: at the IG stage, they show the greatest and lowest levels at ZT 20 and ZT 12, respectively; at the MG stage they peak at ZT 12 to ZT 16; at the RR stage, *preLeGRP1a–c* expression is almost constant over the day (Fig. 5*a–c*).

With respect to the alternative spliced forms, the levels of *asLeGRP1a–c* are lower than the other forms and exhibit little variation in almost all stages and times analysed, with the exception of *asLeGRP1a* and *asLeGRP1b*, which show increased expression at ZT 12 and ZT 20 at the MG stage, respectively (Fig. 5*a–c*).

At the protein level, the highest immunoreaction occurs concomitant with the highest levels of mature *LeGRP1a* and/or *LeGRP1b* forms in all developmental stages analysed (Fig. 5). SDS-PAGEs showing the loading control of crude extracts are shown in Fig. S3. The immunoreactive band of lower electrophoretic mobility displays the highest intensity at ZT 4 at the IG and MG stages and at ZT 8 at the RR stage; the immunoreactive band of highest electrophoretic mobility displays the greatest intensity at ZT 0 and ZT 4 at the IG stage, and at ZT 4 and ZT 8 at the MG and RR stages (Fig. 5*d–f*).

To determine whether the variations observed in the accumulation of LeGRP1 are directly influenced by light–dark transition, tomato plants with IG, MG or RR fruits grown under a 16-h light : 8-h dark photoperiod were transferred to continuous light for a 24 h period. Interestingly, the same patterns of expression than those observed during the day–night cycle were observed for the three forms of each *LeGRP1* gene. The peaks of expression for the three forms of each *LeGRP1* gene are shown in Fig. S5. Moreover, fruits under continuous light display similar patterns in their accumulation of LeGRP1 protein than fruits under a light–dark cycle (Fig. 5*g–i*).

Finally, in order to analyse if the time-course changes observed for the LeGRP1 family in the different stages of tomato fruit development was dependent on signals derived from the rest of the plant, fruits harvested at IG, MG or RR were kept in the chamber, continuing the same light–dark period as the whole plant. Harvested fruits were sampled at ZT 0, ZT 4, ZT 8, ZT 12, ZT 16 and ZT 20 of the following light–dark cycle and LeGRP1 was analysed by western blot (Fig. 6). Remarkably, the accumulation patterns of LeGRP1 protein in green fruits detached from the plant (IG, MG) were similar to those of fruits attached to the plant (Fig. 6*a*, *b* *vs.* Fig. 5*d*, *e*). In contrast, in RR fruits detached from the plant, the pattern of the LeGRP1 protein level was quite different from that observed for RR fruits attached to the plant (Fig. 6*c* *vs.* Fig. 5*f*).

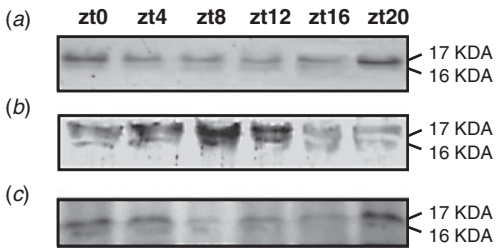

Fig. 6. LeGRP1a–c protein cycling in *S. lycopersicum* fruits harvested from the vine at (*a*) the immature green, (*b*) mature green and (*c*) RR stages. Immunodetection of LeGRP1 was carried out using fruits harvested from the plant and kept in the chamber continuing the same light–dark period as the whole plant for 24 h. Harvested fruits were analysed at Zeitgeber time (ZT) 0, ZT 4, ZT 8, ZT 12, ZT 16 and ZT 20 of the following light–dark cycle after separation from the plant. Western blot analysis was conducted with antibodies raised against LeGRP1a. Thirty μg of protein extracts were loaded. Immunoreactive bands of 16 kDa and 17 kDa are shown.

LeGRP1a–c *expression levels during storage of harvested fruit at different temperatures*

LeGRP1 was also analysed in harvested tomato fruits stored at 4°C or 20°C. In tomato fruits stored at 4°C (7D4 and 7D4 + 2), all *LeGRP1a–c* forms exhibited similar levels to those of MG fruits, with the exception of *mLeGRP1a*, which was higher in 7D4 (Fig. 7*a*). Also, the intensity of the two immunoreactive bands of LeGRP1 at 7D4 and 7D4 + 2 was similar to that seen at the MG stage (Fig. 7*b*).

After 9 days at 20°C (9D20), most *LeGRP1a–c* forms were expressed at higher levels than at the MG or RR stage (Fig. 7*a*). The only exception was *asLeGRP1a*, which was undetectable (Fig. 7*a*). Similarly to the transcript levels, western blot analysis shows an induction in the LeGRP1 protein level when tomatoes are stored for 9 days at 20°C (Fig. 7*b*). Densitometric analysis of both immunoreactive bands reveals increases of ~20-fold with respect to the intensity of each band at the MG stage.

Correlation analysis of the levels of different transcriptional forms of LeGRP1a–c

The levels of the different *LeGRP1a–c* forms obtained during the time-course experiment were used for Spearman correlation analysis (Fig. S6). Positive correlations among the levels of the three *preLeGRP1a–c* genes are found in the green stages analysed, IG and MG (Fig. S6*a*, *b*); however, in the RR stage, only the levels of *preLeGRP1b* correlated positively with *preLeGRP1c* (Fig. S6*c*). Interestingly, negative correlations were observed among all pre-mRNA forms (*preLeGRP1a–c*) and *asLeGRP1c* in IG fruits, and between *mLeGRP1b* and *preLeGRP1c* in MG fruits. In IG fruits, the level of *mLeGRP1b* correlated positively with the levels of *asLeGRP1a* and *mLeGRP1c*; in MG and RR fruits, the levels of *mLeGRP1a* and *asLeGRP1a* exhibited the same correlation. Also, in MG fruits, a positive relation between *mLeGRP1a* and *preLeGRP1b* was observed. Finally, in RR fruits, *preLeGRP1a* had positive correlations with *mLeGRP1a*, *mLeGRP1c* and *asLeGRP1b*, and *mLeGRP1a* had positive

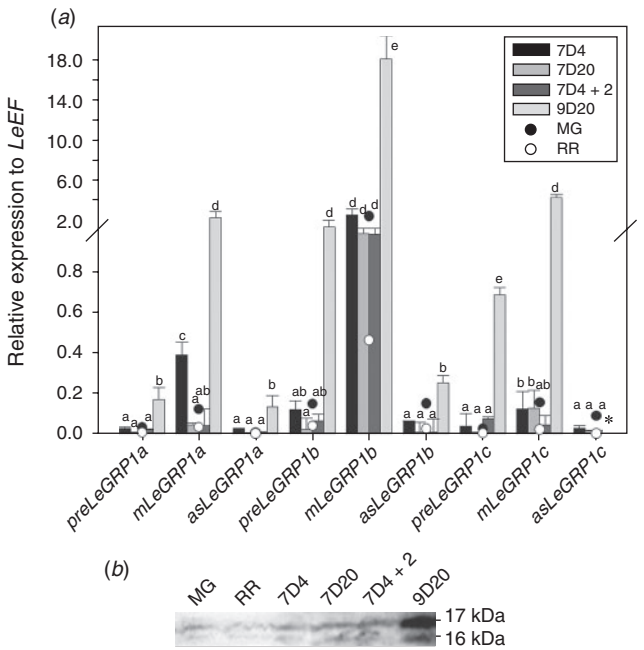

Fig. 7. Expression analysis of the different *LeGRP1a–c* forms and proteins in mature green (MG) and ripe red (RR) tomato fruit stored at different temperatures. Fruits harvested at the MG stage were kept at 4°C for 7 days (7D4) or at 20°C for 7 (7D20) or 9 days (9D20). Cold-stored fruits were transferred to 20°C for 2 days (7D4 + 2). Sample collection was performed at Zeitgeber time 0. (*a*) *LeGRP1a–c* premature, mature, alternative spliced mRNA were quantified by quantitative real-time PCR. The means of the results obtained using three independent mRNAs as template are shown. The *y*-axis refers to the fold difference in a particular transcript level relative to the C_t values of *LeEF1α*; s.d. is shown. For each transcript, bars with the same letters are not significantly different among the treatments ($P < 0.05$). Asterisks (*) indicate no amplification. Black and white circles indicate the level of each transcript in MG and RR fruits, respectively. (*b*) Western blot analysis was conducted with antibodies raised against LeGRP1a. Thirty μg of protein extract was loaded for the immunoblot analysis. Immunoreactive bands of 16 kDa and 17 kDa are shown.

correlations with *asLeGRP1a*, *mLeGRP1b*, *mLeGRP1c* and *asLeGRP1b*.

In silico *analysis of* LeGRP1a–c *promoters*

The promoters of the three *LeGRP1a–c* genes (1 kb upstream of the ATG) and the 5′UTR were analysed *in silico* by using the PLACE signal scan search software (Higo *et al*. 1999). In the *LeGRP1a* promoter, boxes necessary for circadian rhythms in tomato such as *CIACADIANLELHC* (Piechulla *et al*. 1998) and *EVENINGAT* (Harmer *et al*. 2000) have been detected (Fig. S7). The box *CIACADIANLELHC* is also present in the *LeGRP1c* promoter, whereas *CIACADIANLELHC* and *EVENINGAT* are found in the *LeGRP1b* promoter but at greater distances (1.2 and 1.9 kb from the ATG initiation codon, Fig. S7). *STKST1* boxes that drive guard cell expression have been detected in all *LeGRP1* promoters (Plesch *et al*. 2001). Other boxes detected in *LeGRP1a* and *LeGRP1c* promoters are *GCCCORE* (found in many pathogen-responsive genes), *I BOX* (a conserved sequence for light-regulated genes, Terzaghi and Cashmore 1995), *MYBLEPR* (defence-related expression; Chakravarthy *et al*. 2003) and *POLLEN1LELAT52* (pollen-specific activation; Bate and Twell 1998).

Discussion

LeGRP1a–c show distinct circadian oscillations in tomato fruit depending on the developmental stage

In the present work, three *S. lycopersicum LeGRP1* genes, as well as three different products of each gene corresponding to the unspliced pre-mRNA, the mature mRNA and the alternative spliced mRNA (*preLeGRP1a–c*, *mLeGRP1a–c* and *asLeGRP1a–c*, respectively) were studied in tomato fruit. Protein sequences derived from the *mLeGRP1a–c* transcripts display conservation of structure with other known GRPs. Since the alternative spliced products contain an in-frame termination codon within the first half of the intron, they are predicted to encode for 5- to 7-kDa polypeptides comprising only the RNP2 moiety of the RRM (Fig. 1). Probably, these truncated polypeptides do not interact with the RNA target sites in a productive manner, since an intact RNP1 is needed (Schöning *et al*. 2007; Kwak *et al*. 2011). Also, alternative spliced RNA products are candidates for the nonsense-mediated decay pathway, as was found for *AtGRP7* (Heintzen *et al*. 1997; Schöning *et al*. 2007).

By using different *E. coli* mutants, we demonstrate that LeGRP1a–c contain RNA chaperone activity and contributes to enhance cold tolerance (Fig. 2). The fact that LeGRP1a–c were found in the nucleus of tomato fruit (Fig. 4) suggests that these proteins may have a function as a RNA chaperone in this compartment and may influence the export of mRNA from the nucleus to the cytoplasm, as was demonstrated for AtGRP7 (Heintzen *et al*. 1997; Ziemienowicz *et al*. 2003; Kim *et al*. 2007*b*). The correct folding of mRNAs is crucial for the ultimate functions of mRNAs in cells, and RNA chaperones facilitate RNA folding. Spatial and temporal organisation of the nucleus plays a pivotal role in controlling the transcriptome (Shaw and Brown 2004; Spector 2006; Misteli 2007; Chen 2008). Interestingly, LeGRP1a to-c–GFP fusion proteins formed speckles in the nucleus (Fig. 4), similar to those detected for COP1, a negative controller of light input to the clock (Yang *et al*. 2005; Yu *et al*. 2008), PIF3 or Phytochromes (Kircher *et al*. 2002; Bauer *et al*. 2004). Hence, the speckles observed for LeGRP1a–c might represent the site where, in conjunction with other nuclear factors, LeGRP1 proteins may play a role in the nucleus (Kircher *et al*. 2002; Yang *et al*. 2005; Yu *et al*. 2008; Chen 2008).

The levels of the different *LeGRP1a–c* forms, as well as the corresponding immunoreactive GRP proteins, were dependent on the developmental stage of the fruit, decreasing from IG to RR (Fig. 3). In addition, the expression of the *LeGRP1a–c* forms is controlled by a circadian rhythm, which leads to circadian cycling of the LeGRP1 immunoreactive protein (Fig. 5). In this respect, boxes involved in circadian rhythm were detected in the promoters of the three genes (Fig. S7). Interestingly, the type of cycling depended on the developmental stage of the fruit, with peaks of the immature forms at midday in MG fruit and late in IG fruit, and with peaks of mature forms at early morning in IG

fruit and at midday in MG and RR fruit (Fig. 5). Moreover, positive correlations of expression among the three immature forms of *LeGRP1* were detected in practically all the developmental stages analysed (Fig. S6).

LeGRP1a–c levels are modulated by the storage temperature after harvest

Plant GRPs are involved in post-transcriptional RNA processing in response to various stress conditions; for example, *AtGRP7* and *AtGRP8* are constitutively expressed in *A. thaliana* but are rapidly upregulated in response to cold, freezing, thawing and oxidative stress (Kim *et al*. 2005; Schmidt *et al*. 2010). In contrast, in fruits, studies on the biological functions of GRPs under stress conditions are still limited. The results from this study indicate that the different *LeGRP1a–c* forms, as well as GRP immunoreactive proteins, are drastically increased when tomato fruits ripen off the vine for 9 days at 20°C (Fig. 7). However, this increase in GRP is prevented by incubation at 4°C for 7 days. Moreover, GRP levels do not increase even when the cold-treated fruits are transferred to 20°C for 2 days (Fig. 7). Thus, the data indicate that in tomato fruits, GRPs are regulated not only by developmental signals but also by external factors such as temperature. Considering that the analysis of the extended promoter regions (3 kb) of the *LeGRP1* genes does not reveal the presence of cold responsive elements (Fig. S7), it may be possible that post-transcriptional regulation processes might be involved in the modulation of GRP levels at low temperatures. In this respect, temperature changes could produce altered RNA folding that may perturb the splicing or editing machinery interactions.

Do circadian rhythms occur in fruits?

Circadian rhythms have been observed in a wide range of organisms, from cyanobacteria to mammals, and appear to be almost ubiquitous among eukaryotes. In plants, the circadian system regulates a vast range of processes, including changes in calcium levels, photosynthetic activity and leaf movements. In *A. thaliana*, AtGRP7 and -8 have been shown to oscillate, with AtGRP7 peaking at the evening and AtGRP8 at midday (Heintzen *et al*. 1997; Streitner *et al*. 2008). Moreover, AtGRP7 and -8 have been pointed out as part of the output signal of the circadian system, controlling the level of rhythmic transcripts by post-transcriptional regulation (Staiger 2001; Schöning *et al*. 2007; Streitner *et al*. 2008).

In the present work, it has been shown for the first time that LeGRP1a–c display circadian rhythms in fruits, and that the rhythm depends on the developmental stage of the fruit. Therefore, these results indicate that LeGRP1 oscillation is also operational in organs other than leaves, and that these rhythms are affected by developmental and external signals. In agreement, core clock components and some output genes were also found to cycle in developing maize ears and in soybean seeds (Hayes *et al*. 2010; Hudson 2010). In tomato fruits, the accumulation of mRNAs encoding photosynthetic proteins is regulated by a developmental program during a defined period of tomato fruit formation. However, fluctuations in the mRNA levels during day–night cycles are superimposed on this developmental program (Piechulla and Gruissem 1987; Piechulla 1988; Taylor 1989; Facella *et al*. 2008).

The generation of *LeGRP1* mutants or overexpressing plants is needed to definitively prove the functioning of LeGRP1 in the generation of circadian physiological processes. However, homology to other GRPs and correlation analysis among different spliced *LeGRP* forms (Fig. S6) suggest that LeGRP1a–c might transfer their rhythmic activity to other transcripts by means of RNA processing. In this sense, it is very interesting to analyse which signals are implicated in GRP oscillation. Light or the input of photosynthates such as sugars, which modulate fruit gene expression, are good candidates. Sugars are not only the principal carbon fuel but also affect the expression of diurnally regulated genes (Blasing *et al*. 2005; Rogers *et al*. 2005; Hudson 2010). IG tomatoes are the main sink of photosynthates, which inevitably experience diurnal rhythms. In the green stages of fruit development, the input of sugars translocated from the leaves is complemented by the intrinsic photosynthetic activity within the tomatoes themselves. In this sense, the fact that tomato fruits at green stages maintain LeGRP1 fluctuation even when they are detached from the plant suggests that the light signal may be superimposed with respect to the input of photosynthates (Fig. 6). On the other hand, in RR fruits, which lack photosynthetic capacity (Smillie *et al*. 1999), signals derived from the plant seem to be the main contributors of the circadian rhythm (Fig. 6). Another input pathway that regulates the clock is the temperature (which regulates also the cold response pathway) by modulating the gene expression and alternative splicing of clock genes (Espinoza *et al*. 2008). Direct connections between the tomato fruit rhythms and photosynthate uptake, photosynthesis, light and temperature – if any – remain to be elucidated.

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