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Correspondence and requests for materials should be addressed to Y.H. (yiguo.hong@hznu.edu.cn; yiguo.hong@warwick.ac.uk)

* These authors contributed equally to this work.

Tuning *LeSPL-CNR* expression by SlymiR157 affects tomato fruit ripening

Weiwei Chen[1]*, Junhua Kong[1]*, Tongfei Lai[1]*, Kenneth Manning[2], Chaoqun Wu[1], Ying Wang[1], Cheng Qin[1], Bin Li[1], Zhiming Yu[1], Xian Zhang[1], Meiling He[1], Pengcheng Zhang[1], Mei Gu[3], Xin Yang[2], Atef Mahammed[2,4], Chunyang Li[2], Toba Osman[2,4], Nongnong Shi[1], Huizhong Wang[1], Stephen Jackson[2], Yule Liu[5], Philippe Gallusci[6] & Yiguo Hong[1,2]

[1]Research Centre for Plant RNA Signaling, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China, [2]School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK, [3]Clinical Sciences Research Institute, University of Warwick, Coventry CV2 2DX, UK, [4]Department of Botany, Faculty of Agriculture, Fayoum University, Fayoum 63514, Egypt, [5]MOE Key Laboratory of Bioinformatics, Centre for Plant Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China, [6]UMR Fruit Biology and Pathology, University of Bordeaux, INRA, Villenave d'Ornon 33883, France.

In plants, microRNAs (miRNAs) play essential roles in growth, development, yield, stress response and interactions with pathogens. However no miRNA has been experimentally documented to be functionally involved in fruit ripening although many miRNAs have been profiled in fruits. Here we show that SlymiR157 and SlymiR156 differentially modulate ripening and softening in tomato (*Solanum lycopersicum*). SlymiR157 is expressed and developmentally regulated in normal tomato fruits and in those of the Colourless non-ripening (*Cnr)* epimutant. It regulates expression of the key ripening gene *LeSPL-CNR* in a likely dose-dependent manner through miRNA-induced mRNA degradation and translation repression. Viral delivery of either pre-SlymiR157 or mature SlymiR157 results in delayed ripening. Furthermore, qRT-PCR profiling of key ripening regulatory genes indicates that the SlymiR157-target *LeSPL-CNR* may affect expression of *LeMADS-RIN*, *LeHB1*, *SlAP2a* and *SlTAGL1*. However SlymiR156 does not affect the onset of ripening, but it impacts fruit softening after the red ripe stage. Our findings reveal that working together with a ripening network of transcription factors, SlymiR157 and SlymiR156 form a critical additional layer of regulatory control over the fruit ripening process in tomato.

MicroRNAs (miRNAs) are approximately 21-nucleotide (nt) regulatory RNAs that are processed by the dicer-like nuclease DCL1 from stem-loop structures of longer pre-miRNA precursors[1]. An increasing number of miRNAs have been identified in plants and most of them are conserved between species, although species-specific functional miRNAs exist[2–6]. MiRNAs play essential roles in diverse developmental processes and plant responses to biotic and abiotic stresses through suppression of target gene expression by miRNA-guided mRNA cleavage, translational repression or transcriptional RNA silencing[2,3]. One of the characteristics of plant miRNAs is that each miRNA family that is conserved among dicots and monocots has a conserved complementary target site in a mRNA, indicating that these miRNAs may have a similar function in different plants[1,2,6].

Tomato (*Solanum lycopersicum*) is an economically important fleshy fruit and a major worldwide crop with high nutritional value to human health. However tomato becomes edible only after the fruit ripens. Current understanding of the genetic and molecular mechanisms involved in fruit ripening mainly comes from studies of various rare ripening tomato mutants including *ripening inhibitor* (*rin*) and *Colourless non-ripening* (*Cnr*)[7,8]. The *rin* locus encodes a MADS-box transcription factor (TF) LeMADS-RIN[9], while the *Cnr* locus harbours an SBP-box gene, *LeSPL-CNR*, which belongs to the *SQUAMOSA* Promoter Binding Protein-like (*SPL)* TF family[10]. The 3′-UTR of the *LeSPL-CNR* mRNA possesses a potential miRNA156/7 target site, revealing the first clue that miRNAs may control the expression of a crucial ripening TF and thus have a functional role in fruit ripening in tomato. Many miRNAs and small RNAs have been profiled in fruits[11–16], however no miRNA has been experimentally documented to be functionally involved in fruit ripening. Here we report differential roles of SlymiR157 and SlymiR156 in tomato fruit ripening and softening.

Results

SlymiR157 and SlymiR156 are closely related, but only SlymiR157 is perfectly complementary to the potential miRNA target site in the *LeSPL-CNR* mRNA (Supplementary Fig. 1). We analysed the expression profiles of both miRNAs in fruits of normal *Ailsa Craig* (AC) and epi-mutant *Cnr* and found that expression of SlymiR156 was similar in AC and mutant fruits at 40 (breaker) and 54 days post anthesis (dpa). In contrast, SlymiR157 was differentially and developmentally expressed with a relatively higher level in the *Cnr* mutant compared to normal fruits (Fig. 1a, b). These data suggest that SlymiR157 and SlymiR156 may be involved in the regulation of the *LeSPL-CNR* expression at different stages of tomato development, but SlymiR157 may mainly contribute to fruit ripening. This idea is supported by previous findings that transgenic overexpression of SlymiR156 does not obviously affect fruit ripening but instead has significant impacts on fruit formation and yield[17], which is incompatible with the current model generated from RNA deep sequencing and bioinformatics predictions that suggest SlymiR156 is the main small RNA effector controlling ripening in tomato[12,16].

We have cloned the *SlyMIR157* gene through genome walking. Pre-SlymiR157 derived from *SlyMIR157* was predicted to form stem-loop structures (Fig. 1c). Mining of the tomato whole genome sequence[18] indicates that a single copy of *SlyMIR157* is presented and located on tomato Chromosome 3 at 61,724,365–61,724,483 nt. To examine the role of SlymiR157 in ripening we exploited "miRNA-based virus induced gene silencing" (dubbed *Mr* VIGS), to express pre-SlymiRNA in tomato fruits. Pre-SlymiR157 was cloned into Potato virus X (PVX)- and Tobacco rattle virus (TRV)-based VIGS vectors[19,20] to produce PVX/pre-SlymiR157 and TRV/pre-SlymiR157 (Fig. 2a, b). Pre-SlymiR157 was delivered into more than 100 fruits at various days post anthesis from the two different VIGS vectors and 10-20% of injected fruits developed sectors with delayed ripening (DR), whilst several dozens of fruits mock-treated with TE buffer or injected with empty PVX or TRV ripened normally (Fig. 2c, d). However the phenotypes induced by pre-SlymiR157-based *Mr* VIGS were considerably weaker than the non-ripening phenotypes caused by siRNA-based VIGS (dubbed *Sir* VIGS) of *LeSPL-CNR* in fruits injected with PVX/mLeSPL-CNR (Fig. 2e, f), indicating miRNA-mediated regulation of *LeSPL-CNR* gene expression may be a fine tuning mechanism in the control of ripening.

To correlate the DR phenotype with the viral delivery of pre-SlymiR157, we performed qRT-PCR analysis on pre-SlymiR157 and mRNAs of *LeSPL-CNR* and several other key ripening-associated TFs including *LeMADS-RIN*[9], *LeHB1*[21], *SlAP2a*[22] and *SlTAGL1*[23] in three randomly collected fruits injected with PVX/pre-SlymiR157 (Fig. 3a, b, c). Viral pre-SlymiR157 accumulated to a significant high level and was much more abundant in the DR than red-ripe (RR) sectors of all fruits tested. These results suggest that PVX was able to efficiently deliver pre-SlymiR157 into fruits and that the occurrence and amount of pre-SlymiR157 were correlated with the DR phenotype. Consistent with the DR phenotype and massive accumulation of pre-SlymiR157, the level of *LeSPL-CNR* mRNA decreased by 22–50% in DR sectors compared to that in RR tissues of all injected fruits (Fig. 3a, b, c). Such an inverse relationship suggests that miRNA-mediated cleavage of *LeSPL-CNR* mRNA occurs in DR fruit tissues. A similar trend in the mRNA levels of key ripening TF genes *LeHB1*, *SlAP2a* and *SlTAGL1* was also observed although *LeMADS-RIN* mRNA decreased significantly only in the DR sector of two out of three tested fruits (Fig. 3a, b, c). It is worthwhile noting that the four ripening TF genes contain no SlymiR157 target site[18], suggesting that expression of these TF genes could not be directly regulated by SlymiR157 during fruit ripening in tomato.

We also cloned the mature SlymiR157 and SlymiR156 into the PVX-based VIGS vector[19] to produce PVX/SlymiR157 and PVX/SlymiR156 (Supplementary Fig. 2a). They were injected separately into the carpopodium of approximately 100 AC fruits at various stages of development on different trusses on the same plant and on different plants. About 15% of AC fruits injected with PVX/SlymiR157 produced green sectors that showed delayed ripening and later these sectors turned yellow, a phenotype characteristic of

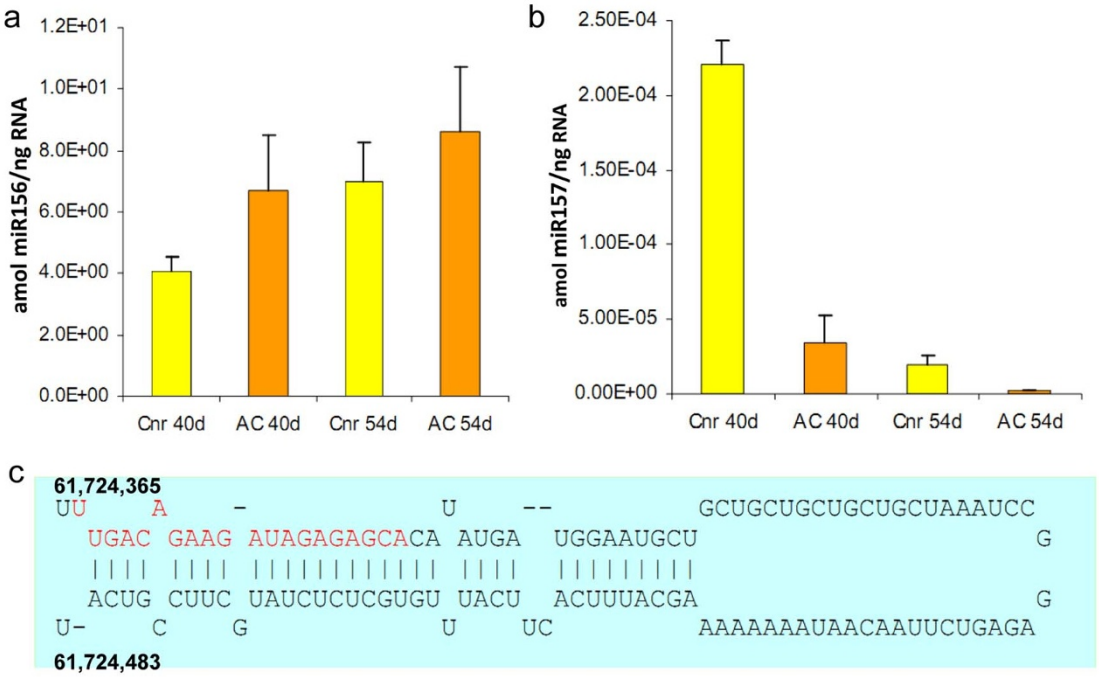

Figure 1 | Differential and developmental regulation of SlymiR157 in tomato fruits. (a) Transcript profiles of SlymiR156 in wild type Ailsa Craig (AC) and *Cnr* epimutant fruits at breaker (40 days post anthesis, dpa) and at 54 dpa (d). (b) Differential and developmental expression of SlymiR157 in AC and *Cnr* fruits. The amount of SlymiR157 was significantly higher at 40 dpa than 54 dpa in *Cnr* fruits. A similar trend of the SlymiR157 level was also found in AC and *Cnr* fruits, but expression of SlymiR157 decreased markedly in AC than *Cnr* fruits at the same stage of the ripening process. (c) Stem-loop structure of the pre-SlymiR157 RNA molecule. The genome coordinates on tomato Chromosome 3 are indicated.

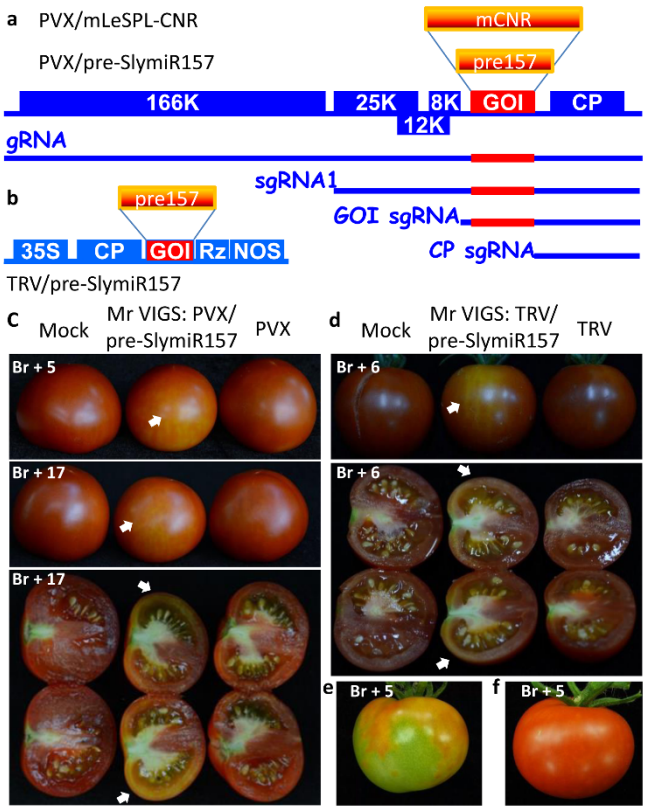

Figure 2 | Pre-SlymiR157 mediated *Mr* VIGS inhibits fruit ripening. (a) PVX-based vectors PVX/pre-SlymiR157 (pre157) for *Mr* VIGS and PVX/mLeSPL-CNR with a non-translational mutant *LeSPL-CNR* (mCNR) RNA for *Sir* VIGS. Genome organization (166K, 25K 12K, 8K and coat protein (CP)) and genomic (g), sub-genomic (sg) RNA1, gene-of-interest (GOI) sgRNA and PVX coat protein (CP) sgRNA of PVX are indicated. (b) TRV-based vector TRV/pre-SlymiR157 for *Mr* VIGS. Transcription of the recombinant TRV, GOI and a ribozyme (Rz) RNA is under the control the 35S promoter and NOS terminator. (c, d) Viral ectopic expression of pre-SlymiR157 from PVX (c) or TRV (d) delayed AC fruits to ripen. Ripening delayed sectors and pericarp in dissected fruits are green or yellow (arrow). Mock- or empty vectors (PVX or TRV)-treated AC fruits ripened normally. (e) *Sir* VIGS of *LeSPL-CNR* by PVX/mLeSPL-CNR developed non-ripening green sectors. 10-20% injected fruits showed the non-ripening phenotypes. (f) AC fruit injected with empty *Sir* VIGS vector PVX ripened normally. Photographs were taken at 5, 6, or 17 days after breaker (Br +5, Br + 6, or Br + 17).

Cnr mutant (Supplementary Fig. 2b). All control fruits that were mock-injected or injected with PVX alone ripened normally. These effects mirrored those obtained using pre-SlymiR157 described above. Fruits injected with PVX/SlymiR156 ripened normally until the breaker stage, however these fruits then quickly softened and became liquefied (Supplementary Fig. 2c). These findings suggest that SlymiR157 and SlymiR156 play different roles in the modulation of ripening and softening, respectively. The softening phenotype remains to be elucidated. However, considering the high expression of SlymiR156 (Fig. 1a) and a significant decrease of *LeSPL-CNR mRNA* after the breaker stage[10], *LeSPL-CNR* may also have a previously unrecognised function as a repressor of softening. Thus, SlymiR156-guided *LeSPL-CNR* mRNA cleavage[12,16] would release such suppression and enhance fruit softening in tomato especially at the later stages of ripening.

On the other hand, viral delivery of mature SlymiR157 was readily detected in all treated fruits. Consistent with the DR phenotype, a higher level of PVX:SlymiR157 was present in the DR than RR sector even though overall levels were low (Fig. 3d, e, f). Unexpectedly, the accumulation of the transcript of the SlymiR157 target *LeSPL-CNR* gene, as well as of *LeMADS-RIN*, *LeHB1*, *SlAP2a* and *SlTAGL1* mRNAs was also found to be greater in the DR than RR sectors of all fruits tested. Although miRNA-mediated posttranscriptional activation has been reported in quiescent mammalian cells and immature *Xenopus laevis* oocytes[24,25], to our knowledge, this is the first example of miRNA-mediated up-regulation of gene expression in plants.

The increased accumulation of *LeSPL-CNR* by SlymiR157 is inconsistent with the phenotype in DR sectors where ripening was delayed (Supplementary Fig. 2b). To explain this discrepancy, we hypothesise that SlymiR157 may regulate tomato ripening via miRNA-mediated mRNA degradation or translational suppression through a dose-dependent mode (Fig. 3). Presence of a massive amount of SlymiR157 could effectively trigger miRNA-mediated cleavage and reduction of the *LeSPL-CNR* mRNA, leading to ripening-delayed phenotype as observed in DR sector of fruits injected with PVX/pre-SlymiR157 and TRV/pre-SlymiR157 (Fig. 2c, d; Fig. 3a, b, c). Conversely, the much lower amount of SlymiR157 below a threshold level would not cause degradation of *LeSPL-CNR* transcript but only represses translation of LeSPL-CNR protein, resulting in maintenance of fruits at breaker stage for a prolonged period during which *LeSPL-CNR* would be constantly transcribed[10]. Consequently, *LeSPL-CNR* mRNA accumulated to a higher amount, as found in the DR than RR sector of fruits injected with PVX/SlymiR157 (Supplementary Fig. 2b; Fig. 3d, e, f), and some of these mRNAs could remain translatable to produce an increasing amount of the LeSPL-CNR protein. The latter would result in up-regulation of the other ripening TF gene expression (Fig. 3d, e, f).

To test whether SlymiR157 could cause translation repression, we developed a "miRNA target – GFP reporter expression" assay in *Nicotinana benthamiana*. The SlymiR157-target site (Tar), wild-type and mutated SlymiR157 sequences were cloned into PVX/GFP[19] to produce PVX/SlymiR157Tar::GFP, PVX/SlymiR157::GFP and PVX/mSlymiR157::GFP (Fig. 4a). RNA transcripts produced by *in vitro* transcription from the three recombinant viruses as well as from PVX/GFP were able to cause systemic infection of *N. benthamiana* plants. GFP protein was readily detected by direct UV visualization of green fluorescence in plants that were infected with PVX/GFP, PVX/SlymiR157::GFP or PVX/mSlymiR157::GFP. However, the insertion of the short RNA of the target site of SlymiR157 totally blocked GFP translation in PVX/SlymiR157Tar::GFP-infected plant (Fig. 4b, c, d, e). These observations are consistent with western blot detection of GFP and PVX coat protein in these plants (Fig. 4f). Importantly, semi-qRT-PCR assays showed that the levels of GFP mRNA produced from all recombinant viruses were similar (Fig. 4g). Due to the conserved and identical nature of both *N. benthamiana* miR157 and SlymiR157 (Supplementary Fig. 1b), we interpret these data to mean that the endogenous miR157 present in the tobacco leaf cells is down-regulating Tar::GFP protein levels through translation repression rather than via miRNA-mediated mRNA degradation. Furthermore, tomato fruits with pedicel injection of PVX/SlymiR157::GFP showed sectors of *Cnr* mutant phenotype whilst fruits injected with PVX/mSlymiR157::GFP, PVX/SlymiR157Tar::GFP or mock treatments ripened normally (Fig. 4h, i, j, k). Taken together, our results imply that SlymiR157 may provide an additional layer of regulation to fine-tune *LeSPL-CNR* expression through a combined action of miRNA-mediated degradation and translation repression in order to control tomato fruit ripening.

Discussion

In tomato, miRNAs are known to be involved in leaf and flower development, yield production, stress response and interactions with pathogens[17,26–29]. High throughput sequencing and bioinformatic analyses predict the presence of many ripening-related small RNAs

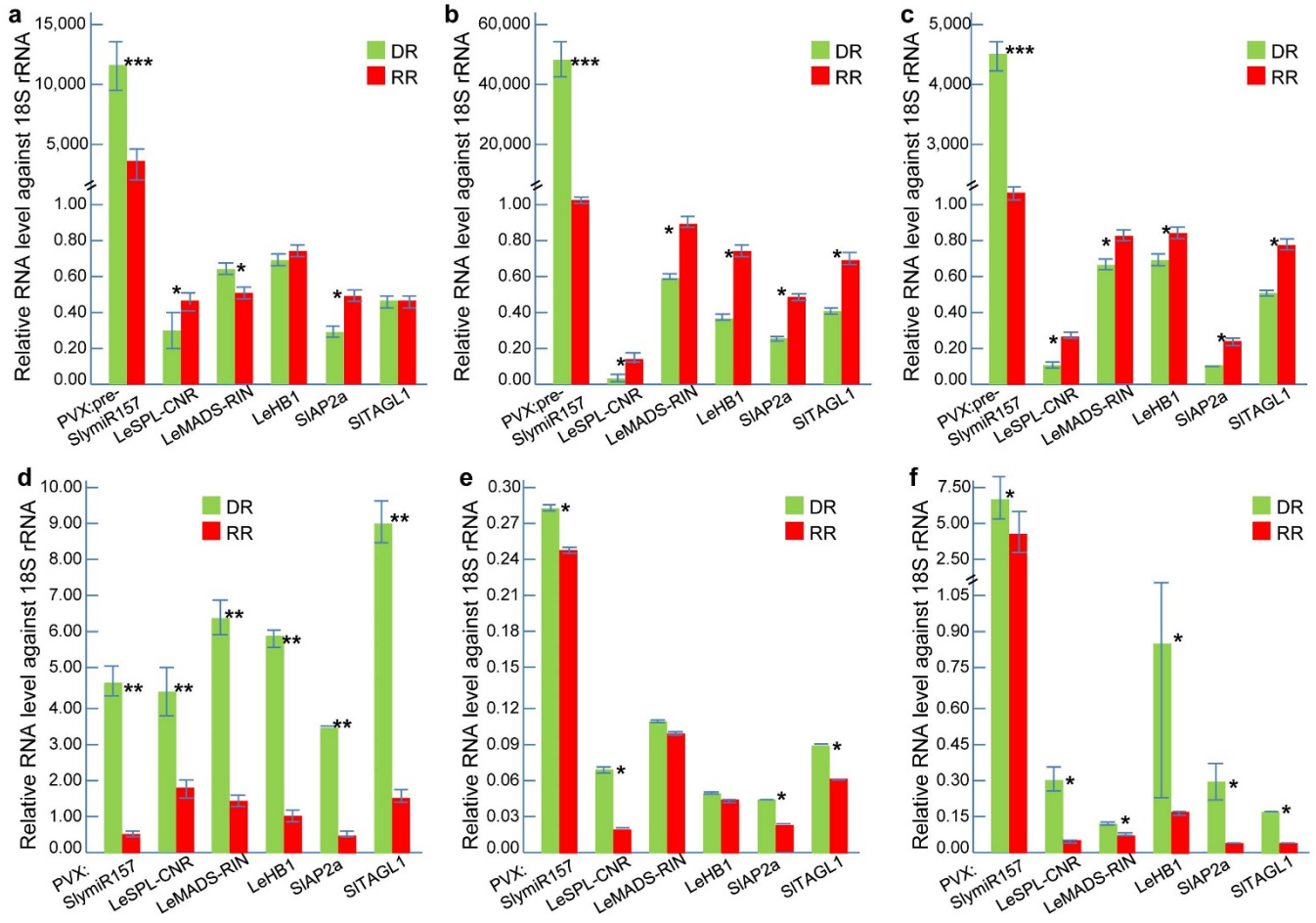

Figure 3 | ***Mr* VIGS affects expression of *LeSPL-CNR* and key ripening TF genes.** (a-f) Viral delivery of pre-SlymiR157 and mature SlymiR157, as well as expression of the SlymiR157 target gene *LeSPL-CNR* and TF genes (*LeMADS-RIN*, *LeHB1*, *SlAP2a* and *SlTAGL1*) in delayed ripening (DR) and red-ripe (RR) sectors of three different AC fruits injected with PVX/pre-SlymiR157 (a, b, c) or PVX/SlymiR157 (d, e, f) were respectively analysed by qRT-PCR. Relative levels of RNA transcripts were obtained by normalising against the baseline expression levels of the internal control 18S rRNA. qRT-PCR of each biological sample was performed in 3-15 technical replicates. Student's ***t***-tests were carried to evaluate the statistical significance at the level of $p \leq 0.001$ (***), 0.01 (**) or 0.05 (*) for each of the specific RNA transcripts between DR and RR sectors of each different biological sample. Although there is variance, the results of these qRT-PCR assays show a similar correlation between the viral delivery of pre-SlymiR157 or mature SlymiR157 and the mRNA levels of ripening TF genes in the DR and RR sectors of fruits tested.

and miRNAs and their targets in fruits at various developmental stages[11–16]. For instance, SlymiR172 is predicted to cleave *SlAP2a* mRNA and several miRNAs are also believed to target ripening-associated genes coding for pectate lyase (SlymiR482), endo-1, 4-ß-glucanase (SlymiR395), ß-galactosidase (SlymiRZ7), ethylene-insensitive 2 (SlymiR828), serine/threonine protein kinase (SlymiR1917) and 1-aminocyclopropane-1-carboxylic acid synthase (SlymiR159)[16]. Nevertheless, there is no experimental evidence to demonstrate that any of these miRNAs have a functional role in tomato fruit ripening.

It has also been postulated that SlymiR156 may target and cleave *LeSPL-CNR* mRNA and affect ripening in tomato[12,16]. However transgenic over-expression of SlymiR156 in a previous report[17], and viral delivery of SlymiR156 described in this study, clearly show that SlymiR156 does not significantly affect ripening per se, but that it mainly influences fruit softening (Supplementary Fig. 2) and other developmental processes. Further, a more recent study reports that miR156 regulates tomato ovary and fruit development[30]. Transgenic over-expression of precursor of the Arabidopsis miR156 (AtmiR156b) in tomato produced abnormal flowers and fruits with extra carpels and meristem-like structures inside the ovaries. Development of such ectopic organs is associated with miR156-mediated regulation of SPL/SBP box TF gene expression as well as with alternations of expression of TFs that are essential for meristem maintenance and reproductive development[30].

However, SlymiR157 is differentially expressed in normal tomato fruits and the *Cnr* epi-mutant and regulates *LeSPL-CNR* expression. Moreover, viral delivery of either pre-SlymiR157 or its mature form by VIGS vectors can delay ripening in tomato. Thus, SlymiR157 may provide a post-transcriptional mechanism for fine-tuning *LeSPL-CNR* expression and protein levels through miRNA-induced mRNA cleavage or/and translation repression, possibly in a dose-dependent manner to affect *LeSPL-CNR* function. Furthermore, the coordinated gene expression profiles of *LeSPL-CNR, LeMADS-RIN*, *LeHB1*, *SlAP2a* and *SlTAGL1* also suggest that *LeSPL-CNR* may act as a positive upstream regulator for these other four TFs (Fig. 5). This is consistent with previous findings that LeMADS-RIN protein interacts with promoters of several key ripening-associated TFs including LeHB1 and LeSPL-CNR, and the latter is required for LeMADS-RIN to bind its target promoter sequence in the transcriptional control of ripening[31]. Thus, our findings support and greatly extend a model for the control of ripening involving a network of TFs[7,8,32], and a critical extra layer of regulation involving miRNAs.

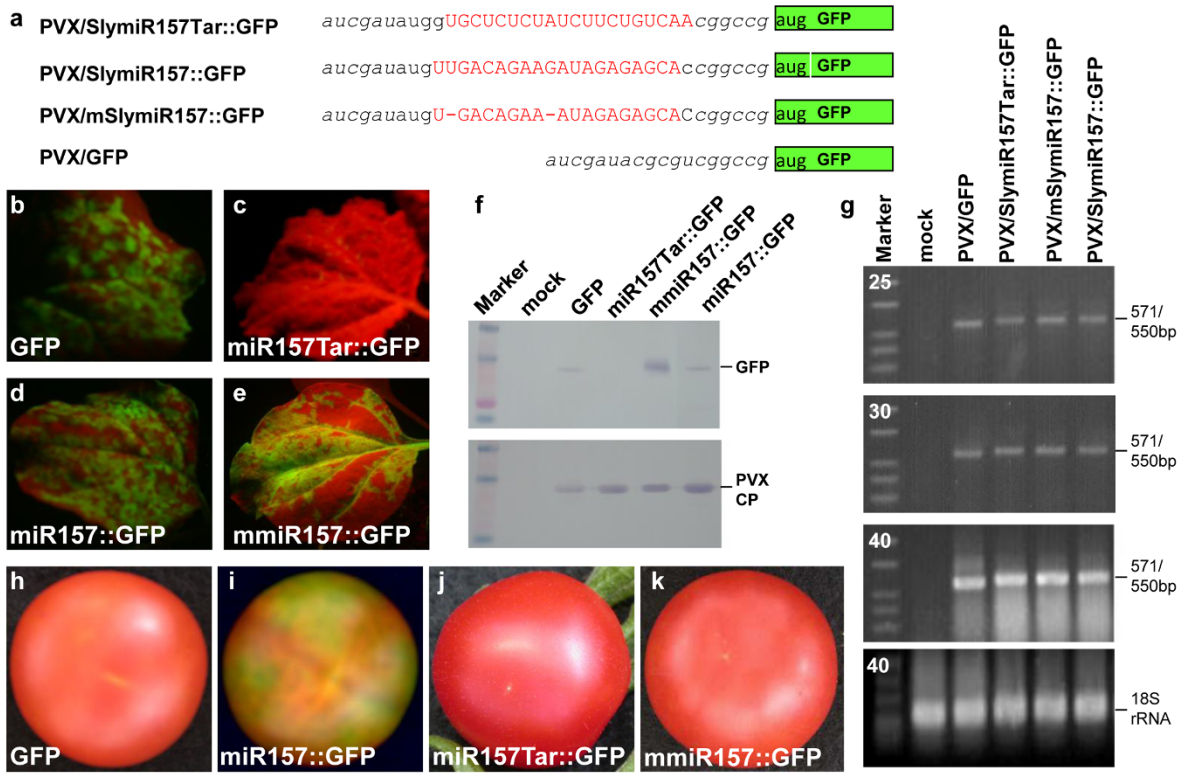

Figure 4 | miRNA target – GFP reporter expression assay. (a) PVX-based miRNA target – GFP reporter expression vectors. (b-e) *Nicotinana benthamiana* leaves were inoculated with PVX/GFP (GFP, b), PVX/SlymiR157Tar::GFP (miR157Tar::GFP, c), PVX/SlymiR157::GFP (miR157::GFP, d) or PVX/mSlymiR157::GFP (mmiR157::GFP, e). Photographs were taken under long-wavelength UV light at 7 days post inoculation. GFP fluorescence is green and leaf auto-fluorescence shows red. (f) Western blot detection of GFP and PVX coat protein (CP) in mock- or PVX-inoculated leaf tissues using antibody raised specifically against GFP and the viral CP. Positions of GFP and PVX CP are indicated. (g) Detection of recombinant PVX::miRNA target::GFP RNA in same leaf samples as used in Western blot by semi-qRT-PCR. The positions and sizes of amplified fragments and the number of PCR cycles are indicated. 18S rRNA was used as controls. (h-k) AC fruits were injected with PVX/GFP (h), PVX/SlymiR157::GFP (i), PVX/SlymiR157Tar::GFP (j) or PVX/mSlymiR157::GFP (k). Non-ripening sectors show green. Fruits were photographed at 7 days after breaker.

Methods

Plant materials and growth conditions. Tomato (*Solanum lycopersicum* cultivar Ailsa Craig) and *Cnr* epimutant fruits were grown in a growth room with a photoperiod of 16-hr at 25°C during daytime and 20°C at night. Plants were grown to three-five trusses. *Nicotiana benthamiana* plants were grown under the same conditions as for tomato. Tomato fruits at various days post anthesis and *N. benthamiana* leaves at 7 days post-inoculation were collected and kept at −80°C until use.

Genome walking experiments. Genome walking to clone the *SlyMIR157* gene was carried out as described[10]. The stem-loop structure of pre-SlymiR157 was predicted using an online mfold web server (mfold.rit.albany.edu).

Construction of vectors for *Mr* and *Sir* VIGS. The pre-SlymiR157 fragment was RT-PCR amplified using two sets of primers, digested with restriction enzymes and cloned into the potato virus X (PVX)- and tobacco rattle virus (TRV)-based VIGS vectors[19,20] to produce PVX/pre-SlymiR157 and TRV/pre-SlymiR157, respectively (Supplementary Table 1). For construction of PVX/SlymiR157, PVX/SlymiR156, PVX/SlymiR157Tar::GFP, PVX/SlymiR157::GFP, PVX/mSlymiR157::GFP, a pair of relevant complementary primers (Supplementary Table 1) was mixed in TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) buffer, heated at 95°C for 3 minutes, then cooled to room temperature to allow them to anneal to form double-strand forms which were cloned into either PVX or PVX/GFP vector[19]. The *Sir* VIGS vector PVX/mLeSPL-CNR carrying a mutated *LeSPL-CNR* gene was constructed as described[10]. All constructs were verified by sequencing.

***In planta* miRNA functional assays and *in vitro* molecular analysis.** *Mr* and *Sir* VIGS and the miRNA target – GFP reporter expression assay were performed as described[10,19,20]. Absolute qRT-PCR to quantify SlymiR157, relative qRT-PCR and semi-qRT-PCR to measure viral delivery of pre- and mature miRNAs, TF and GFP reporter transcripts were performed as described[10,19,32] using primers listed in Supplementary Table 2. Direct visualization of GFP green fluorescence in leaves and western blot detection of GFP and PVX coat protein were carried out as described[19,32].

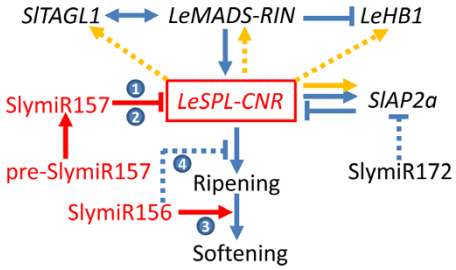

Figure 5 | Involvement of miRNAs in modulation of tomato fruit ripening. A transcription factor (TF) gene network including *LeSPL-CNR*, *LeMADS-RIN*, *LeHB1*, *SlAP2a* and *SlTAGL1* is involved in tomato ripening. Blue arrows or "**T**" signs denote interactions among these TF genes that have been previously demonstrated[7,8,32]. Yellow arrows indicate that *LeSPL-CNR* may activate expression of the ripening TFs, as described in this work. SlymiR172 is proposed to down-regulate *SlAP2a* and affect ripening but this idea is not supported by any *in planta* experimental evedience[7]. SlymiR157 (pre-SlymiR157) may cause ① miRNA-guided mRNA degradation or ② translation repression to affect *LeSPL-CNR* function depending on whether a high above-threshold (①) or low below-threshold (②) levels of SlymiR157 are present. The correlation between the high expression of SlymiR156 (Fig. 1a) and a significant decrease of *LeSPL-CNR mRNA*[10] after the breaker stage suggests that apart from being an activator for the early ripening, *LeSPL-CNR* may also have a previously unrecognised function as a repressor of softening. Thus, SlymiR156-guided *LeSPL-CNR* mRNA cleavage[12,16] would enhance fruit softening at the later stage of tomato development (③) in addition to its weak role in the epigenetic modulation of fruit ripening[17] (④).

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Author contributions

W.C., J.K. and T.L. designed and performed *Mr* and *Sir* VIGS and qRT-PCR; K.M. cloned and analysed SlymiR157; C.W., Y.W., C.Q., B.L., Z.Y., X.Z., M.H., P.Z. performed *Mr* and *Sir* VIGS and molecular analysis. M.G., X.Y., A.M., C.L., T.O. and N.S. performed translation repression assays; H.W., S.J., Y.L. and P.G. were involved in analysis of data and helped writing the paper. Y.H. initiated the project, analysed data and wrote the paper.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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