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Virus-induced gene complementation reveals a transcription factor network in modulation of tomato fruit ripening

SUBJECT AREAS:

PLANT SCIENCES

GENE REGULATION

TRANSCRIPTION

VIROLOGY

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Plant virus technology, in particular virus-induced gene silencing, is a widely used reverse- and forward-genetics tool in plant functional genomics. However the potential of virus technology to express genes to induce phenotypes or to complement mutants in order to understand the function of plant genes is not well documented. Here we exploit *Potato virus X* as a tool for virus-induced gene complementation (VIGC). Using VIGC in tomato, we demonstrated that ectopic viral expression of *LeMADS-RIN*, which encodes a MADS-box transcription factor (TF), resulted in functional complementation of the non-ripening *rin* mutant phenotype and caused fruits to ripen. Comparative gene expression analysis indicated that *LeMADS-RIN* up-regulated expression of the SBP-box (SQUAMOSA promoter binding protein-like) gene *LeSPL-CNR*, but down-regulated the expression of *LeHB-1*, an HD-Zip homeobox TF gene. Our data support the hypothesis that a transcriptional network may exist among key TFs in the modulation of fruit ripening in tomato.

* These authors contributed equally to this work.

In plants, annotations of the dicot *Arabidopsis* and the monocot rice genome sequences have predicted around 30,000–40,000 genes in the respective genomes^{1,2}. Meanwhile, the high throughput and vast capacity of next-generation sequencing have tremendously increased the scope of whole genome sequences. High-quality, or draft, whole genome sequences of a number of higher plant species such as *Arabidopsis*¹, rice², maize³, potato⁴ and tomato⁵ are already publically available. These data together with bioinformatic predictions, as well as transcriptome and proteome analyses of the expression patterns of thousands of unknown genes, will certainly lead to the identification of a large number of putative genes with no designated biological functions. Forward and reverse genetics are two classical approaches to define gene function⁶. In practice, both approaches involve mutant analysis that provides a genetic link between genotype and phenotype. In plants, mutants can either be identified in natural populations or induced by chemical mutagenesis. Techniques including physical mutagenesis, T-DNA insertion, transposon integration and RNAi-mediated knockdown are also used to create mutants in order to assign gene function, however these genetics tools are largely restricted to model plants such as *Arabidopsis*, tobacco or rice⁶. Definite proof of gene function often involves complementation in a mutant background, which is usually achieved through stable transformation⁶. However, transformation is time consuming and technically challenging in many crops of economic importance. Thus to elucidate the biological function of genes predicted from whole genome sequences remains an arduous task and there is a need to develop novel functional genomics tools in order to bridge the knowledge gap from gene-to-function⁶.

Plant virus technology does not require transformation and it has attracted attention because it is an effective tool to delineate gene function in plants. In particular, virus-induced gene silencing (VIGS) has become a rapid reverse- and forward-genetics tool in functional genomics since the early development and refinement of VIGS vectors based on plant viruses including *Tobacco mosaic virus*, *Potato virus X* (PVX) and *Tobacco rattle virus*^{7–10}. VIGS has now been applied to more than 30 plant species across the angiosperm phylogeny^{11,12}. For instance, we

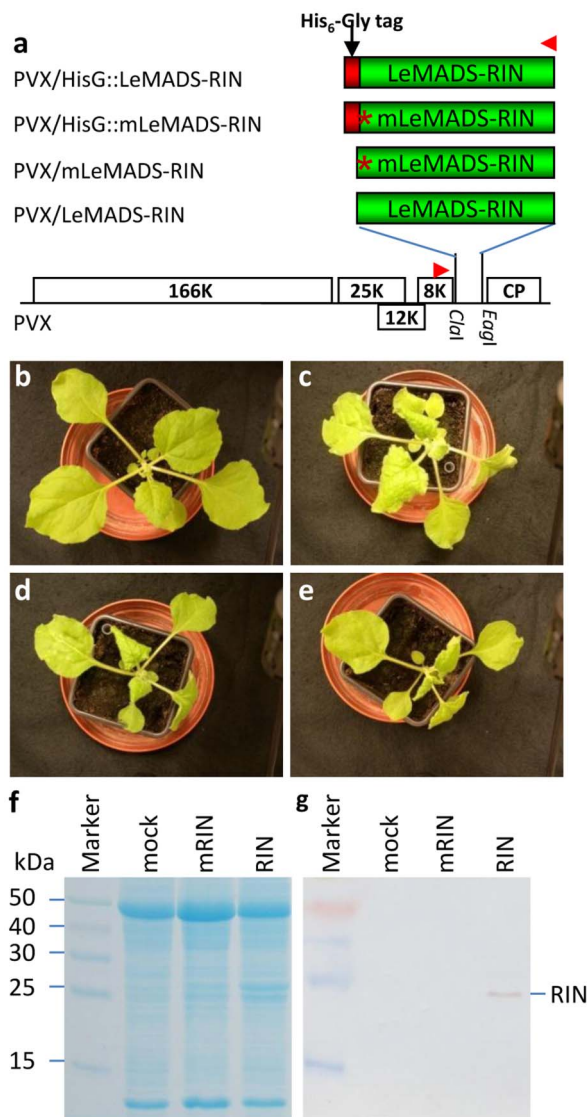


Figure 1 | Ectopic expression of *LeMADS-RIN* from VIGC vectors in *Nicotiana benthamiana*. (a) Construction of VIGC vectors. The PVX vector is shown together with the wild-type *LeMADS-RIN* and the mutated *mLeMADS-RIN* (asterisked; Table S1) used to create PVX/*LeMADS-RIN*, PVX/*mLeMADS-RIN*, PVX/*HisG::LeMADS-RIN* and PVX/*HisG::mLeMADS-RIN* vectors. PVX/*LeMADS-RIN* and PVX/*HisG::LeMADS-RIN* were designed to express either free *LeMADS-RIN*, or the *LeMADS-RIN* protein tagged with six consecutive histidine residues and one glycine residue (*His6-Gly*), respectively. Viral RNA dependent RNA polymerase (166K), movement proteins (25, 12 and 8K) and coat protein (CP) as well as the *His6-Gly* tag are indicated. The cloning sites and the position of primers (triangles) used for RT-PCR detections of the PVX::*LeMADS-RIN* RNA are also indicated. (b–e) Mock-inoculated *N. benthamiana* (b) remained healthy while plants infected with PVX/*LeMADS-RIN* (c), PVX/*HisG::LeMADS-RIN* (d), PVX/*HisG::mLeMADS-RIN* (e) or PVX/*mLeMADS-RIN* (data not shown) developed chlorosis and mosaic phenotypes, characteristic of PVX infection. Photographs were taken at 14 days post-inoculation (dpi). (f, g) Western blot detection of the *LeMADS-RIN* protein. Proteins were extracted from leaves of plants infected with PVX/*HisG::LeMADS-RIN* (RIN), PVX/*HisG::mLeMADS-RIN* (mRIN), or from mock-inoculated (mock) plants at 14 dpi. The blot was probed with an antibody specific for the *His6-Gly* polypeptide tag (g). A coomassie blue-stained SDS-PAGE gel (f) shows equal loading of samples (5 µg total soluble protein). The sizes of the molecular mass standards (marker) and position of the *LeMADS-RIN* protein (RIN) are indicated on the left and right, respectively.

have applied a PVX-based VIGS to inhibit the expression of SBP-box *LeSPL-CNR* and HD-Zip homeobox *LeHB-1* transcription factor (TF) genes in order to reveal their essential roles in the control of fruit ripening and floral organogenesis in tomato^{13,14}. In addition to VIGS, virus expression technology can also be used for high level production of enzymes, green fluorescent protein and foreign proteins of pharmaceutical and industrial value, and for studying the biological function of heterologous genes in plants^{15–19}. More recently we have reported the development of a novel virus-based RNA mobility assay for use in elucidating biological relevance of systemic florigen signalling in flower induction^{20,21}, as well as a movement-deficient virus system to dissect intercellular RNA silencing^{22–25}. However, the potential of virus technology to express endogenous genes to induce phenotypes, or to complement mutants, in order to investigate their functions in plants is not well recognised.

The tomato *ripening inhibitor (rin)* locus encodes the MADS-box TF *LeMADS-RIN*, which is induced at the onset of ripening²⁶. The *LeMADS-RIN* gene resides on chromosome 5 adjacent to *MACROCALYX (MC)*, the tomato *APETALA1* orthologue. The *rin* mutation is caused by a spontaneous deletion of the 3'-end of the *LeMADS-RIN* gene and approximately 1 kb of the intergenic sequence between *LeMADS-RIN* and *MC*. A *MADS-RIN/MC* transcript is expressed in the *rin* mutant and the chimeric RNA cannot produce functional *LeMADS-RIN* and *MC* proteins. Elegant genetic and molecular analyses confirm that the *LeMADS-RIN* gene alone is responsible for ripening, providing the first insight into the dedicated fruit-specific transcriptional modulation of ripening²⁶. The tomato *LeHB-1*, a class-I homeodomain leucine zipper TF protein, is involved in fruit ripening through transcriptional regulation of ACC oxidase gene expression¹⁴. Another key player in fruit ripening is the gene at the tomato *Colourless non-ripening (Cnr)* locus. *Cnr* is a pleiotropic ripening mutant with colourless fruits and an altered texture resulting from loss of cell-to-cell adhesion. The gene underlying this mutation was identified as a *SQUAMOSA* Promoter Binding Protein (SBP)-box gene (*LeSPL-CNR*), belonging to the *SPL* gene family of TFs¹³. There are several other TFs, which have also been demonstrated to play a role in the control of tomato fruit ripening²⁷, however the regulatory network of these ripening-associated TFs remains to be elucidated. Here we exploited a PVX-based viral expression vector to express *LeMADS-RIN* for virus-induced gene complementation (VIGC) in tomato *rin* mutant. We demonstrated that expression of *LeMADS-RIN* from VIGC vectors enabled functional complementation and caused *rin* fruits to ripen. Our data also favour a presumption that a regulatory network in which *LeMADS-RIN* up-regulates *LeSPL-CNR* whilst down-regulating *LeHB-1* may play a role in the modulation of fruit ripening in tomato.

Results

Viral ectopic expression of *LeMADS-RIN* from VIGC vectors.

In order to express *LeMADS-RIN* in plant leaf tissues and tomato fruits, the coding region for *LeMADS-RIN* was PCR amplified and cloned into the PVX-based gene expression vector²⁸ to produce PVX/*LeMADS-RIN* (Fig. 1a). To facilitate detection of the *LeMADS-RIN* protein we also generated PVX/*HisG::LeMADS-RIN* in a modified vector PVX/*HisGMF1* (Fig. S1), in which the coding sequence for a *His6-Gly* tag was fused in frame to the 5' end of the *LeMADS-RIN* gene. As negative controls, a stop codon was introduced to replace the start codon of *LeMADS-RIN* and this non-sense mutated gene was cloned into the PVX and PVX/*HisGMF1* vectors to produce PVX/*mLeMADS-RIN* and PVX/*HisG::mLeMADS-RIN* (Fig. 1a; Fig. S1). The integrity of the cloned genes was confirmed by sequencing.

To test whether the *LeMADS-RIN* protein could be efficiently expressed from the PVX-based VIGC vectors, infectious RNA transcripts were produced by *in vitro* transcription from PVX/*LeMADS-RIN*, PVX/*mLeMADS-RIN*, PVX/*HisG::LeMADS-RIN*, PVX/*HisG::mLeMADS-RIN*.

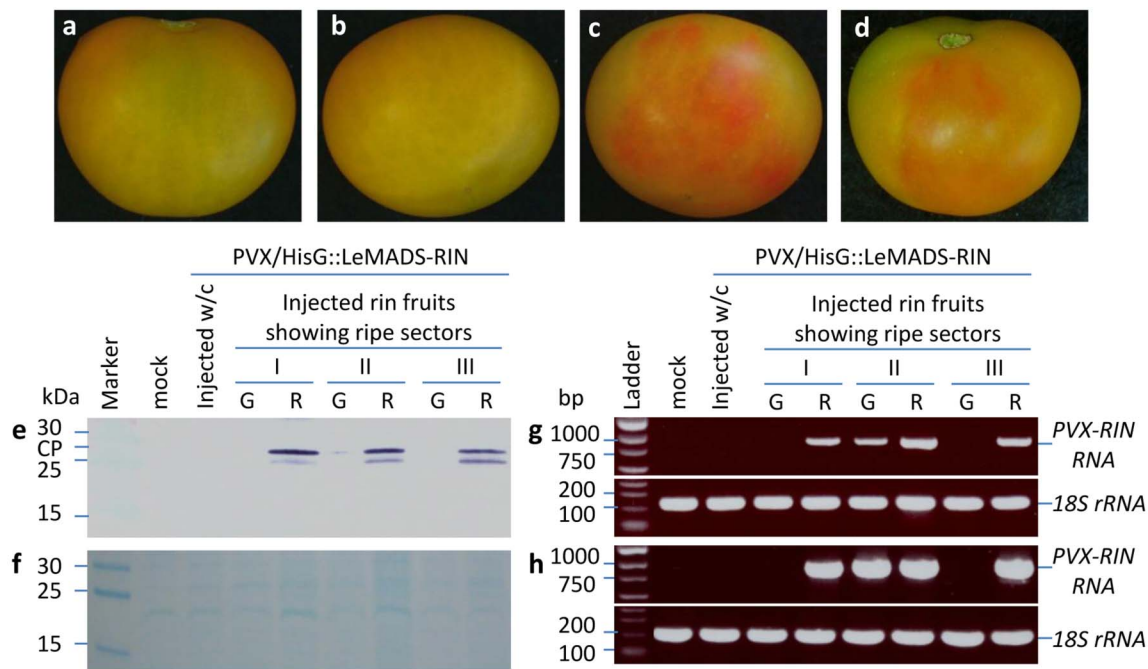


Figure 2 | VIGC-mediated complementation of ripening in *rin* mutant fruits. *Rin* fruits were needle-injected with TE buffer (a), or with PVX/HisG::mLeMADS-RIN (b), PVX/LeMADS-RIN (c) and PVX/HisG::LeMADS-RIN (d). Only *rin* fruits injected with VIGC vector PVX/LeMADS-RIN or PVX/HisG::LeMADS-RIN which are capable of expressing LeMADS-RIN or His6-Gly tagged LeMADS-RIN, respectively, resulted in ripening. Mock-injected fruits or those injected with PVX, PVX/mLeMADS-RIN or PVX/HisG::mLeMADS-RIN from which no LeMADS-RIN protein could be expressed remained green and non-ripe. Fruits were photographed at 3 weeks post-injection (wpi). (e) Presence of VIGC vectors in *rin* fruits. Western blot analyses of total protein extracted from *rin* fruits at 3 wpi of the VIGC vector indicated that PVX CP accumulated in ripe red sectors (R), but little to none in the green sectors (G) of three *rin* fruits (I–III) individually injected with PVX/HisG::LeMADS-RIN. No viral CP was detected in *rin* fruits that were mock-injected (mock) or injected with the VIGC vector but showed no complementation of ripening (Injected w/c). (f) A coomassie blue-stained SDS-PAGE gel shows equal loading of samples (10 µg total soluble proteins). The positions and sizes of the molecular mass standards (marker) are indicated. (g, h) Semi-quantitative (sq) RT-PCR assays of transient viral expression of *LeMADS-RIN*. Total RNA was extracted from *rin* fruits as described in (e) at 3 wpi and used for sqRT-PCR with 25 (g) and 30 (h) cycles of amplification. Equal amount of total RNA (100 ng) used in each of the sqRT-PCR reactions was verified by detecting a similar level of 18S rRNA in all samples. Viral ectopic expression of the *LeMADS-RIN* mRNA is consistent with presence of the VIGC vectors in the *rin* fruits. The positions and sizes of the 1-kb DNA marker (Ladder) and positions of RT-PCR products specific for PVX::LeMADS-RIN mRNA (PVX-RIN RNA) as well as 18S rRNA are indicated.

mLeMADS-RIN or the empty PVX vector, and mechanically inoculated onto *Nicotiana benthamiana*. In contrast to the mock-inoculated plant (Fig. 1b), virus-inoculated plants developed local chlorosis in inoculated leaves at 3–7 days post-inoculation (dpi) and followed later by mosaic and curling in systemic young leaves, typical phenotypes associated with PVX infection (Fig. 1c–e). Systemic young leaf tissues were then collected at 14 dpi for further molecular analysis. Recombinant viral RNAs were detected by RT-PCR and direct sequencing of the RT-PCR products confirmed that the *LeMADS-RIN* RNA was maintained in all VIGC vectors during the course of infection. Moreover, a HisG::LeMADS-RIN protein was readily detected by immunoblotting in plants inoculated with PVX/HisG::LeMADS-RIN, but not in PVX/HisG::mLeMADS-RIN or mock-inoculated plants using an antiserum raised against His₆-Gly tag (Fig. 1f, g). These results clearly demonstrate that the tomato *LeMADS-RIN* protein can be expressed from VIGC vectors in *N. benthamiana*, and over-expression of *LeMADS-RIN* does not have a deleterious effect on plant growth and development.

LeMADS-RIN mediated complementation in *rin* fruits. To investigate the feasibility of VIGC in plant gene function analysis, we tested whether ectopic viral expression of *LeMADS-RIN* could complement the non-ripening phenotype in *rin* mutant. Through the carpocodium (pedicel), immature (approx. 3–4 weeks after anthesis) or mature green fruits were needle-injected with either recombinant PVX RNA transcripts, or leaf saps from *N. benthamiana*

plants mock-inoculated or inoculated with PVX/LeMADS-RIN, PVX/mLeMADS-RIN, PVX/HisG::LeMADS-RIN, PVX/HisG::mLeMADS-RIN, or the empty PVX vector. For each construct, 40–50 *rin* fruits were injected in repeated experiments. Not surprisingly, all mock-treated fruits, and fruits injected with PVX, PVX/mLeMADS-RIN or PVX/HisG::mLeMADS-RIN remained non-ripe, showing characteristics of the *rin* mutant phenotype (Fig. 2a, b). However nearly 40% of fruits injected with PVX/LeMADS-RIN or PVX/HisG::LeMADS-RIN appeared to ripen at 2–3 weeks after virus-injection, evidenced by developing red sectors on the fruit (Fig. 2c, d). Thus, expression of *LeMADS-RIN* or His₆-Gly tagged *LeMADS-RIN* from PVX/LeMADS-RIN or PVX/HisG::LeMADS-RIN respectively, can functionally complement the *rin* mutant.

PVX/LeMADS-RIN, PVX/mLeMADS-RIN, PVX/HisG::LeMADS-RIN, PVX/HisG::mLeMADS-RIN or PVX were initially delivered into fruits via needle-injection of the carpocodium. However all viruses were able to spread through the plants during the course of the experiment. Consequently, newly grown young systemic leaves developed chlorosis and mosaic and curling phenotypes typically associated with PVX infection. Nevertheless, virus-positive plants, similar to virus-free plants, still had the competence to flower and produce tomato fruits. Remarkably, in plants systemically infected with PVX/LeMADS-RIN or PVX/HisG::LeMADS-RIN, approximately 30% of the newly developed (but non-injected) fruits ripened, producing red ripe sectors as shown in Fig. 2c–d. These data suggest that viruses can move from the injected carpocodium through the phloem transportation system into

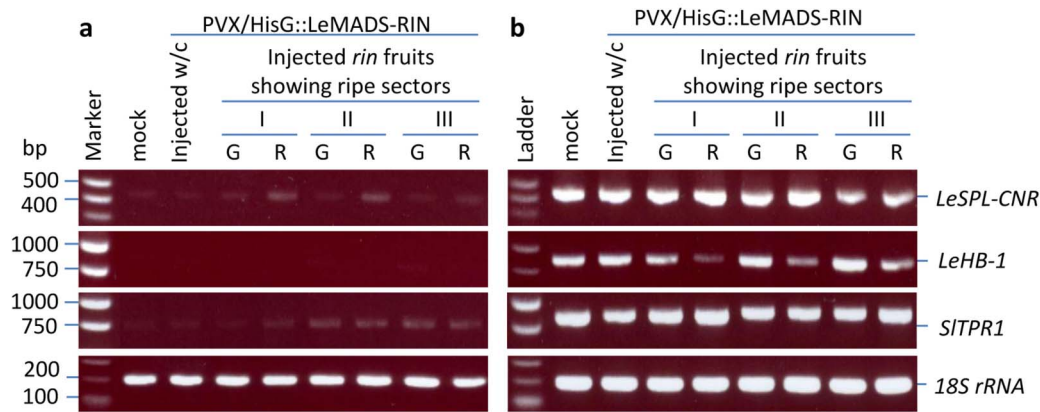


Figure 3 | Impact of *LeMADS-RIN* on expression of *LeSPL-CNR*, *LeHB-1* and *SITPR1*. Total RNA was extracted from *rin* fruits as described in Fig. 2e at 3 wpi of the VIGC vectors and used for sqRT-PCR with 25 (a) and 30 (b) cycles of amplification. That an equal amount of total RNA (100 ng) was used in each of the sqRT-PCR reactions was verified by detecting a similar level of 18S rRNA in all samples. The positions and sizes of the 1-kb DNA marker (Ladder) and positions of RT-PCR products specific for *LeSPL-CNR*, *LeHB-1* and *SITPR1* mRNA as well as 18S rRNA are indicated.

fruits where viral ectopic expression of the *LeMADS-RIN* gene can cause *rin* fruit ripening.

To correlate VIGC of fruit ripening with viral ectopic expression of the *LeMADS-RIN* gene, we dissected the pericarps of the ripe (red) and non-ripe (green) sectors of *rin* fruits. Total protein was extracted from the separate ripe and non-ripe tissues, as well as from fruit pericarps injected with PVX/HisG::LeMADS-RIN but showing no complementation, and from that of mock-injected *rin* fruits. Western blot assays showed that the level of the PVX coat protein (CP) was abundant in ripe sectors of *rin* fruits injected with PVX/HisG::LeMADS-RIN. This was evidenced by the fact that a protein of the predicted size of approximately 26 kDa was specifically recognised by an antibody raised against the PVX CP (Fig. 2e) while coomassie blue-stained SDS-PAGE gel shows equal loading of samples (Fig. 2f). There was no accumulation of PVX CP in the mock *rin* fruit, or in the non-complemented fruit injected with PVX/HisG::LeMADS-RIN. On the other hand, in green sectors of the complemented fruits, the level of PVX CP was mostly undetectable or significantly reduced (Fig. 2e, f). Thus, the presence of the VIGC vector PVX/HisG::LeMADS-RIN was consistent with the functional complementation of ripening in the *rin* fruits. However to our surprise, using an anti-His6-Gly antibody we were unable to detect the HisG::LeMADS-RIN protein in the pericarps of ripe sectors. It is possible that a low level of HisG::LeMADS-RIN sufficient to complement the *rin* mutant phenotype was expressed but was beyond the limit of western blot detection. Such low expression could be the result of gene silencing induced by the VIGC vector, or by loss of the inserted *LeMADS-RIN* RNA sequence from the PVX genomic RNA.

We thus used a more sensitive semi-quantitative (sq) RT-PCR approach to detect *LeMADS-RIN* mRNA expressed from the PVX-based VIGC vectors. Total RNA was extracted from ripening-inhibited fruits collected from mock-inoculated *rin* fruits, and from the separate ripe and green non-ripe tissues, as well as from non-complemented fruits at 3 weeks post-injection with PVX/HisG::LeMADS-RIN. Total RNA (100 ng) was used together with a set of primers PP82/PP779 (Fig. 1a; Tables S1 and S2) in one-step sqRT-PCR assays. These primers allowed specific detection of an approximately 900-bp transcript containing partial PVX RNA and the full-length *LeMADS-RIN* mRNA (Fig. 1a). Consistent with western blot assays, no transient viral *LeMADS-RIN* mRNA was detected in pericarps of the control fruits. However PVX::LeMADS-RIN RNA was detected in the red ripening sectors of complemented fruits whilst levels of these RNAs were either undetectable or obviously reduced in the green non-ripening sector of the same fruits (Fig. 2e, f). That an equal amount of total RNA was used in each of the

sqRT-PCR reactions was verified by detecting a similar level of 18S rRNA in all samples using primers PP271 and PP272 (Tables S1 and S2). A similar result was also obtained in complemented *rin* fruits injected with PVX/LeMADS-RIN (Fig. S2). Moreover, the non-translatable *mLeMADS-RIN* mRNA was detectable in fruits which were injected with PVX/*mLeMADS-RIN* or PVX/HisG::*mLeMADS-RIN* and remained non-ripe (Fig. S2). Taken together, these data demonstrate that the ripening phenotypes were caused by *LeMADS-RIN* expression from the VIGC vectors.

***LeMADS-RIN* regulates *LeSPL-CNR* and *LeHB-1* expression.** Complementation of the *rin* mutant through VIGC offers a quick and efficient strategy to directly investigate *LeMADS-RIN*-dependent regulation of ripening-associated genes. To test this idea, we performed comparative sqRT-PCR assays and examined the level of RNA transcripts of *LeSPL-CNR*, *LeHB-1* and *SITPR1* in mock-treated and VIGC fruits. These genes were chosen because they are known to be involved in fruit ripening through direct or indirect regulation of ethylene biosynthesis and signalling transduction^{13,14,29}. The mRNA level of *LeSPL-CNR* was low in mock-injected fruit and in a non-complemented fruit that had been injected with the VIGC vector PVX/HisG::LeMADS-RIN. A similar expression pattern for *LeHB-1* or *SITPR1* gene was also observed (Fig. 3a, b). However sqRT-PCR assays of total RNA extracted from the non-ripe green and ripening red sectors of complemented *rin* fruits injected with PVX/HisG::LeMADS-RIN clearly showed that viral transient expression of *LeMADS-RIN* had different impacts on the expression of the three ripening-associated genes. *LeSPL-CNR* was readily detected in the ripening sectors after 25 cycles of PCR (Fig. 3a), its mRNA level being higher than that in the non-ripening green sectors although such differential expression levels became less obvious after a saturated 30-cycle PCR (Fig. 3b). On the other hand, the mRNA level of *LeHB-1* was constantly lower in ripening red than non-ripe green sectors (Fig. 3a, b). In contrast to both *LeSPL-CNR* and *LeHB-1* TF genes, the level of *SITPR1* expression remained relatively unaffected. A similar level of 18S rRNA was detected in all samples, showing that an equal amount of total RNA (100 ng) was used in each of the sqRT-PCR reactions. Taken into account the *LeMADS-RIN* gene expression levels in these tissues (Fig. 2), these data indicate that *LeSPL-CNR* is up-regulated while *LeHB-1* is down-regulated by *LeMADS-RIN*, but that *LeMADS-RIN* is not directly involved in the regulation of the *SITPR1* gene transcription.

Discussion

In this report we describe a VIGC technology for virus-induced gene complementation and provide ‘proof of concept’ that VIGC works in



defining functions of fruit ripening-related TF genes in the tomato *rin* mutant. Several advantages of VIGC can be envisaged in its application in plant functional genomics. First, VIGC is an easy and quick technique to complement gene function in mutant plants. Ectopic over-expression of plant genes from a VIGC vector results in 'gain-of-function', thus providing a biological link between phenotype and genotype. Second, VIGC possesses a relatively high efficiency for mutant phenotype complementation, although this efficiency could still be improved through optimization of experimental conditions and inoculation protocols. Third, VIGC does not require time-consuming and labour-intensive stable transformation that is associated with transgenic complementation. Fourth, the feasibility of VIGC based on a single plant virus PVX was only tested in the tomato *rin* mutant. Further studies to develop various virus-based VIGC vectors are currently ongoing. However, the rational and potential uses of VIGC should be applicable to other plant and crop species. For gene function analysis in tomato fruit ripening, VIGC offers an additional advantage that ripening and non-ripe tissues can be collected from the same individual fruits. These tissues which are at the precisely equivalent age and stage are most suitable for comparative gene expression analysis.

Tomato is an excellent model crop to investigate fruit development and ripening. Recent advances on the understanding of genetic and molecular mechanisms that control fruit ripening have come from the study of various tomato ripening mutants, such as *rin* and *Cnr*^{27,30}. Some of these mutant loci encode master ripening modulator TFs including LeMADS-RIN and LeSPL-CNR^{13,26}. LeHB-1 and several other TFs have also been demonstrated to play an important role in tomato fruit ripening^{14,31–35}. Recently, it has been reported that expression of *TAGL1* (Tomato AGAMOUS-like 1), a member of the AGAMOUS clade of MADS-box genes, is capable of complementing the *rin* mutant although this contradicts the fact that the *rin* mutant maintains normal *TAGL1* expression without ripening^{33,36}. Nevertheless, the rationale of transcriptional control of fruit ripening is well established. However, whether a functional network exists among ripening-related TFs remains to be elucidated.

Using VIGC, we have demonstrated that ectopic expression of *LeMADS-RIN* resulted in functional complementation of non-ripening phenotypes and caused *rin* fruits to ripen (Fig. 1; Fig. 2). Such complementation was unlikely to be caused by the recombination of homologous RNA sequences. However it could be predicted that actually VIGS would take place rather than VIGC. Having an endogenous gene in the viral genome would be expected to induce VIGS and potentially produce little of the virus-expressed gene product. Thus VIGS probably contributed to our failure to detect the *LeMADS-RIN* protein in *rin* tomato, but had little effect on accumulation of the TF protein in heterologous *N. benthamiana* plants (Fig. 1; Fig. 2). On the other hand viral infection as well as fruit ripening are dynamic processes, VIGS usually occurs after virus establishes infection and PVX is able to evade self-silencing³⁷, therefore PVX-based vectors are suitable to be used for VIGC. In our experiments, the observed complementation effect in the *rin* mutant could derive from a temporary and transient expression of the *LeMADS-RIN* TF. This could occur from the VIGC vectors at the beginning of the viral infection prior to occurrence of VIGS, or from VIGC viral RNA being survived the VIGS, triggering a ripening process which was only observed afterwards. VIGS and off-target gene silencing could be induced by all four VIGC vectors, however complementation was only observed in *rin* fruits injected with PVX/*LeMADS-RIN* or PVX/HisG::*LeMADS-RIN*, but not in any fruits injected with PVX/m*LeMADS-RIN* or PVX/HisG::m*LeMADS-RIN*. Thus this excludes the possibility that VIGS of *LeMADS-RIN* or siRNA-mediated silencing of off-target genes could result in the *rin* complementation.

Our data support the conclusion that *rin* complementation has taken place in a percentage (40%) of injected fruits. VIGS might be

the reason that the remaining 60% of fruits do not show complementation. It is also possible that homologous recombination between the endogenous mutant *rin* transcript and the *LeMADS-RIN* RNA in the VIGC vectors occurred during virus RNA replication in the cytoplasm of cells. In this scenario the modified virus, which could incorporate part of endogenous mutant *rin* RNA sequences, might continue its systemic movement within the tomato plants. This could subsequently lead to VIGC vectors losing their capacity of complementation in fruits although the probability of such type of event is unknown. In addition, instability of RNA inserts in the VIGC viral genomic RNA could have affected complementation efficiency. Partial or even complete elimination of the *LeMADS-RIN* RNA was observed in all PVX constructs (Fig. 3S, Supplementary Text) and that would certainly have a negative influence on the efficiency of VIGC. Moreover, experimental conditions including inoculation protocols, timing and plant growth environments could also affect VIGC.

The *LeMADS-RIN*-mediated VIGC process does not affect the expression of *SITPR1*, which encodes an ethylene receptor-interacting protein and functions in the ethylene signalling transduction associated with fruit ripening²⁹. However, *LeMADS-RIN* transiently expressed from VIGC vectors was able to up-regulate *LeSPL-CNR* expression (Fig. 2; Fig. 2S; Fig. 3), consistent with the observed reduction of *LeSPL-CNR* expression in *rin* mutant²⁷. Our finding suggests that *LeMADS-RIN* is likely to act upstream of *LeSPL-CNR* in a proposed TF network (Fig. 4). On the other hand *LeHB-1* is highly expressed in immature and mature green fruits, but the level of *LeHB-1* mRNA declines during ripening and is maintained at a relatively low level in ripe fruits. *LeHB-1* is involved in transcriptional regulation of ACC oxidase (*LeACO1*) gene expression through its binding to the *LeACO1* promoter. Silencing of *LeHB-1* in tomato fruit greatly reduces (*LeACO1*) mRNA levels and inhibits ripening. However, over-expression of *LeHB-1* causes conversion of floral organs into fruits¹⁴. Thus expression of the homeobox TF *LeHB-1* must be tightly controlled during the process of tomato fruit development and ripening. Consistent with this idea, we found that *LeMADS-RIN* acted as a negative modulator to down-regulate *LeHB-1* transcription in the ripening sector of *rin* fruits (Fig. 3). Taken together, our comparative analysis of gene expression in the ripe and non-ripe sectors of *rin* fruits suggests that *LeMADS-RIN* might link with *LeSPL-CNR* and *LeHB-1* to form a potential TF network in the modulation of fruit ripening (Fig. 4). It is possible that expression changes of *LeSPL-CNR* and *LeHB-1* could just be a consequence of fruit ripening. In this scenario *LeMADS-RIN* might only have an indirect effect on these downstream TF genes. However, direct binding of *LeMADS-RIN* to the promoters of *LeSPL-CNR* and *LeHB-1* has been elegantly demonstrated through various molecular and biochemical assays^{38,39}. Thus our findings, along with experimental data reported in the literatures^{38,39} are in favour of a model in which *LeMADS-RIN* probably exerts a direct impact on *LeSPL-CNR* and *LeHB-1* gene expression in the transcriptional regulation of tomato fruit ripening.

In summary, we have exploited the potential of plant virus technology, namely VIGC, for gene expression to induce phenotypes and to complement gene function in the tomato *rin* mutant. Using VIGC we provide evidence to support the hypothesis that a transcriptional network may exist among three key TFs *LeMADS-RIN*, *LeSPL-CNR* and *LeHB-1* in the modulation of tomato ripening. The function of the master TF *LeMADS-RIN* and its impact on *LeSPL-CNR* and *LeHB-1* expression as well as on ethylene-mediated ripening are shown in a proposed model (Fig. 4). This model is supported by the findings of other research groups that the *LeMADS-RIN* protein can directly bind to the promoters of the *LeSPL-CNR* and *LeHB-1* genes^{38,39}.

Methods

Construction of VIGC vectors. A cDNA clone of the tomato (*Solanum lycopersicum* cultivar Ailsa Craig) *LeMADS-RIN* gene in pK7WGF2 (Zhong, L. & Giovannoni, J.J. unpublished data) was used as a template for construction of all VIGC vectors. It

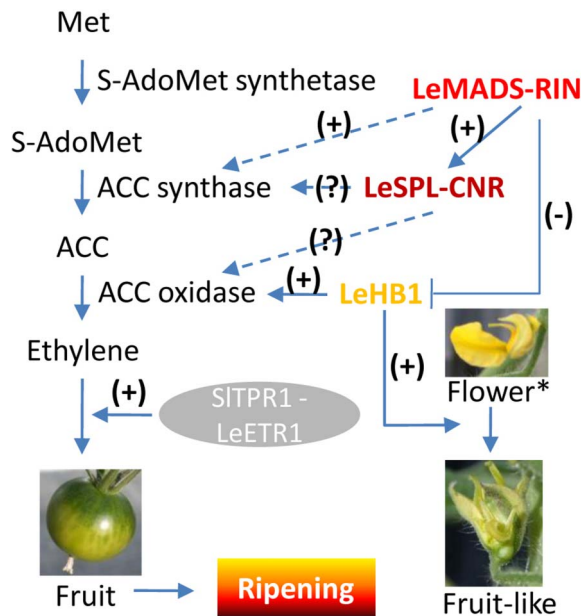


Figure 4 | A regulatory network among key ripening-associated TFs. Methionine (Met) is converted into S-adenosyl methionine (S-AdoMet) by the S-AdoMet synthetase. The key enzyme ACC synthase cleaves the amino butyrate from S-AdoMet to release 1-aminocyclopropane-1-carboxylic acid (ACC). The ACC oxidase then converts ACC to ethylene which acts as an essential phytohormone to trigger fruits to ripen in tomato. The master TF LeMADS-RIN may affect production of ACC synthase and regulate the biosynthesis of ethylene. LeMADS-RIN up-regulates (+) the transcription of *LeSPL-CNR* that may be directly or indirectly involved in the control of ethylene biosynthesis. Meanwhile, LeMADS-RIN serves as a negative TF to down-regulate (−) the *LeHB1* expression at the transcriptional level. The latter is shown to directly modulate the ACC oxidase gene expression and affect ethylene production for fruit ripening. However, over-expression of *LeHB1* also results in abnormal flower (Flower*) and fruit development¹⁴. The *SITPR1* gene product interacts with the ethylene receptor *LeETR1*, which exerts a positive impact on fruit ripening²⁹.

should be noted that *S. lycopersicum* has been recently renamed from *Lycopersicon esculentum* and genes isolated before and after the re-nomenclature contain either *Le* or *Sl* prefix, respectively. The wild-type and mutant *LeMADS-RIN* genes were PCR amplified using *Pfu* DNA polymerase (Promega) and a pair of primers PP797/PP799 or PP798/PP799 (Tables S1 and S2). The resulting PCR products were digested with *Cla*I and *Eag*I, and cloned into the *Cla*I/*Eag*I sites of PVX²⁸ to produce PVX/*LeMADS-RIN* and PVX/*mLeMADS-RIN* respectively (Fig. 1a). The wild-type and non-sense mutant *LeMADS-RIN* genes were also cloned in-frame to the coding sequence of a histidine-glycine (His6-Gly) tag in the *Cla*I/*Eag*I sites of a modified PVX/HisGMF1 vector (Fig. S1) to generate PVX/HisG::LeMADS-RIN and PVX/HisG::mLeMADS-RIN (Fig. 1a). All constructs were confirmed by nucleotide sequencing.

Viral infection and plant maintenance. RNA transcripts were produced by *in vitro* transcription from each of the PVX constructs (Fig. 1) after linearization with *Spe*I, and then were mechanically inoculated onto young leaves of *N. benthamiana* and *S. lycopersicum* cultivar Ailsa Craig plants as described²⁸. Mock inoculation of plants with TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) was used as a negative control. All plants were maintained in an insect-free glasshouse at 25°C with supplementary lighting to give a 16-h photoperiod. Local and systemic symptom developments were assessed on a daily basis and recorded photographically using a Nikon Digital Camera Coolpix990.

VIGC (Virus-induced gene complementation). RNA transcripts of PVX, PVX/*LeMADS-RIN*, PVX/*mLeMADS-RIN*, PVX/HisG::LeMADS-RIN or PVX/HisG::mLeMADS-RIN were needle-injected into the carpododium of *rin* tomato fruits at the mature green stage. Fruits were injected with TE buffer as negative controls. Homogenized saps prepared from systemic leaves of *N. benthamiana* with mock-inoculation or inoculated with PVX, PVX/*LeMADS-RIN*, PVX/*mLeMADS-RIN*, PVX/HisG::LeMADS-RIN or PVX/HisG::mLeMADS-RIN were also used to injected *rin* fruits on different trusses of the tomato plants in repeated experiments (more than three times). Within 2–3 weeks after injection *rin* fruits started to develop

ripening sectors due to VIGC-mediated effect, and they were routinely examined and photographed with a Nikon Coolpix 995 digital camera.

Semi-quantitative RT-PCR (sqRT-PCR). Total RNA was extracted from leaf tissues of *N. benthamiana* and from tomato fruits using an RNeasy Plant Mini Kit (Qiagen). RT-PCR was performed as previously described²⁰ with different pairs of primers PP82/PP83, PP82/PP799, PP83, PP271/PP272, PP298/PP300, PP402/PP403 and PP404/PP405 (Tables S1 and S2) to detect PVX, PVX::LeMADS-RIN RNA, plant 18S rRNA, *LeSPL-CNR*, *LeHB1* and *SITPR1* mRNA, respectively.

Western blotting. To investigate expression of HisG::LeMADS-RIN and viral coat protein (CP) from the VIGC vectors, total protein was extracted from leaf and fruit tissues collected from *N. benthamiana* or tomato as previously described⁴⁰. Western blot analyses of protein aliquots (10 µg) were performed with polyclonal antibodies specifically raised against PVX CP, or an anti-His-Gly tag antibody (Invitrogen), and detected using a goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Sigma) and 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP/NBT) substrates (Roche) as previously described^{37,40}.

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Author contributions

T.Z., H.Z., T.L., C.Q., N.S., H.W., M.J. and S.Z. designed and performed experiments, Z.F., Y.L., Z.W., S.J., J.J.G., D.R. and P.G. contributed through discussions and revised the paper. Y.H. initiated the project, designed the experiments and wrote the paper.

Additional information

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

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