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Brief Communication

Promoting virus-induced gene silencing of pepper genes by a heterologous viral silencing suppressor

Yingjia Zhou[1], Yingtian Deng[1], Dan Liu[1], Hongzheng Wang[1], Xi Zhang[1], Tingting Liu[1], Jubin Wang[1], Yue Li[1], Lijun Ou[2], Feng Liu[2], Xuexiao Zou[2], Bo Ouyang[1] and Feng Li[1,3,*]

[1]*Key Laboratory of Horticultural Plant Biology (MOE), College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan, China*
[2]*College of Horticulture, Hunan Agricultural University, Changsha, China*
[3]*Hongshan Laboratory, Huazhong Agricultural University, Wuhan, China*

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**Correspondence* (Tel 86-27-87280505; fax 86-27-87280505; email chdlifeng@mail.hzau.edu.cn)

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Pepper is an important vegetable cultivated worldwide. Pepper fruits accumulate unique metabolites, capsanthin and capsaicin, which are important raw materials for natural pigment and medicine. Pepper plants are rich in genetic diversity and are attractive subjects for fruit developmental studies. The release of pepper reference genomes provided key genetic information for dissecting function of pepper genes underlying various interesting phenotypes (Kim *et al*., 2014; Qin *et al*., 2014). However, due to the difficulty in transformation, dissecting genetic mechanisms in pepper remained technically challenging. RNA silencing is a small RNA-mediated gene silencing mechanism operating in most eukaryotes and working as a natural antiviral defence mechanism (Ding, 2010). Virus-induced gene silencing (VIGS) not only targets invading virus, but also can repress expression of plant genes, and thus, many viruses were developed into VIGS tools for studying plant gene function. Tobacco rattle virus (TRV) was a well-developed VIGS vector and widely used in plants (Ratcliff and Martin-Hernandez, 2001; Shi *et al*., 2021). TRV-based VIGS was frequently used in pepper, but the efficiency was not optimized, particularly for silencing gene expression in flowers and fruits.

In our initial effort to optimize VIGS efficiency in pepper leaves, we made the pTRV2-GFP-CaPDS vector from the pTRV2-LIC vector (Dong *et al*., 2007), which allowed us visualize TRV spreading and monitor VIGS efficiency (Figure 1a), and determined optimal experimental condition, in which agrobacterium of OD_{600} 0.004 was infiltrated on cotyledon of two-week-old seedlings and resulted 60% of treated plants showing photobleaching. Successful VIGS depends on efficient virus movement in plants, which is controlled impart by antiviral RNA silencing and viral suppressors (VSR) (Li and Ding, 2006). We reasoned that expression of heterologous VSRs from TRV may enhance its spreading in pepper, thus enhancing VIGS efficiency. To test this hypothesis, a panel of pTRV2 vectors were made from pTRV2-GFP-CaPDS with GFP in-frame fused to various VSRs, including citrus tristeza virus CP, P23 and P20, tomato bush stunt virus P19, cucumber mosaic virus 2b (C2b) and tomato aspermy virus 2b (T2b) (Li and Ding, 2006) (Figure 1a). These vectors were co-expressed with pTRV1 in pepper cotyledon by agroinfiltration. Green florescence imaging and Western blot analysis showed that all TRV viruses replicated and spread well in pepper plants (Figure 1b,c). Statistics analysis showed that C2b-expressing vector caused PDS silencing in 93% of treated plants, resulting in highest VIGS efficiency among all vectors (Figure 1b). With the high efficiency TRV2-GFPC2b-CaPDS vector, we screened our pepper germplasm and identified about 30 accessions with PDS silencing efficiency ranging from 70% to 100%. C2b (from subgroup II CMV-Q, X00985) showed the best performance in promoting VIGS efficiency, which is consistent with its three merits: (i) it has strong systemic silencing suppressor activity that promotes virus spreading; (ii) it has a weak local silencing suppressor activity that will not affect silencing of target genes in target cells; (iii) it has short coding sequences that minimizes fitness cost brought to TRV vector (Guo and Ding, 2002). These merits made C2b an idea VSR to promote TRV spreading in pepper and induce target gene silencing in systemically infected leaves.

To test how the optimized TRV vector performs in pepper flowers, pTRV2-C2b-CaAGL vector was made to target pepper *AGAMOS* (*AG*) gene in which GFP ORF was removed to increase virus fitness (Figure 1a). Four pepper accessions with good PDS-VIGS efficiency were infected with pTRV1 and pTRV2-C2b-CaAGL. About 6 weeks after infiltration, altered flower phenotype appeared among all the four pepper accessions, while L085 and L265 accessions showed better overall performance with good flower setting and silencing efficiency (81% and 84% respectively). Persistence of TRV infection in pepper flowers and knocking-down of *CaAG* expression were confirmed by RT-PCR (Figure 1d,e). Close examination showed different types of flower structure on pTRV2-C2b-CaAGL infected pepper, which were different from reported *Arabidopsis ag* null mutant and *RNAi*-lines (Figure 1f) (Bowman and Smyth, 1989; Mizukami and Ma, 1995). VIGS of *AG* in Arabidopsis also resulted in abnormal floral structures that are different from its CRISPR mutant (Figure 1f). Although VIGS of *AG* revealed its dual roles in petal identity and floral determinacy in both species, the floral determinacy in pepper and Arabidopsis appeared different (Figure 1f). These results suggested that our optimized VIGS method can silence genes efficiently in pepper flowers and also revealed unique advantage for VIGS in studying developmental genes.

To test VIGS efficiency in pepper fruit, pTRV2-C2b-CaCCS vector was constructed (Figure 1a), which targets the key gene in capsanthin/capsorubin biosynthesis (Kim *et al*., 2014). Five red pepper accessions (L085, L099, L228, L252 and L265), suitable for long-term maintenance in growth room, were infected with

pTRV1 and pTRV2-C2b-CaCCS by agroinfiltration. The TRV-infected plants were regularly watered and fertilized till fruit maturation, which took 14–16 weeks after infiltration. The results showed that 97% and 100% of pTRV2-C2b-CaCCS infected L085 and L228 plants produced yellow fruits while no plants infected with pTRV2-C2b produced yellow fruits,

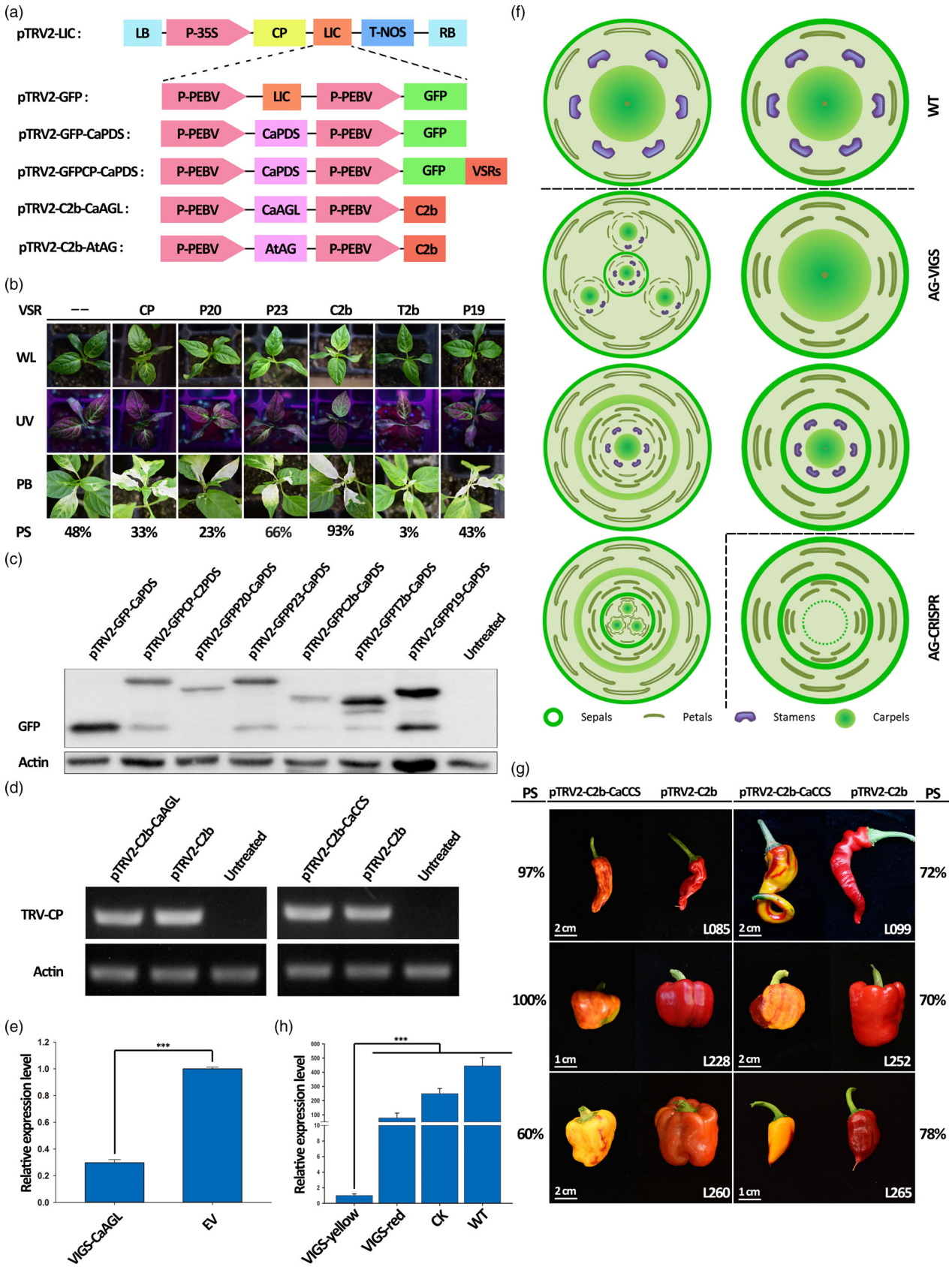

Figure 1 Optimization of tobacco rattle virus (TRV)-mediated virus-induced gene silencing (VIGS) in pepper plants. (a) Structure of TRV vectors. (b) TRV systemic movement and PDS silencing in pepper. WL, white light condition; UV, ultraviolet light condition; PB, photobleaching phenotype; PS, percentage of silenced plants. (c) Western blot detection of GFP protein in systemically infected leaves of plants treated as indicated above each lane. (d) Detection of viral transcript by RT-PCR in pepper flower (left) and fruit (right). (e) Detection of pepper AG transcript levels in pTRV2-C2b-CaAG (VIGS-CaAGL) and pTRV2-C2b (EV) infected flowers. (f) Patterns various floral structures in pepper (left) and Arabidopsis (right). WT, wild-type flowers; AG-VIGS, flowers from VIGS plants; AG-CRISPR, flowers from CRISPR mutant plants, in which the green dashed circle represents repeating pattern of sepal–petal–petal. (g) Silencing of *CaCCS* in pepper fruits. Treatment was indicated on top of pepper images; pepper accession numbers were indicated bottom right. PS, percentage of plants producing *CaCCS* silenced fruits. (h) Detection of *CaCCS* transcript level in various pepper fruit tissue from L099. WT, wild-type red tissue; CK, pTRV2-C2b infected red tissue; VIGS-red, pTRV2-C2b-CaCCS infected red tissue; VIGS-yellow, pTRV2-C2b-CaCCS infected yellow tissue.

indicating high efficiency of *CaCCS* silencing in fruit (Figure 1g). Knocking-down of *CaCCS* transcript levels and persistence of TRV infection in the yellow fruit were confirmed by RT-PCR (Figure 1d, h). These results suggested that the optimized TRV vector and selected pepper accessions could constitute an efficient VIGS system for studying gene function in pepper fruits.

In summary, our study optimized TRV-based VIGS methods in pepper and provided highly efficient vectors and pepper accessions that could facilitate studies of gene function throughout pepper life cycle. And the strategy could also be applied to other plant VIGS system.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

F.L., Y.J.Z. and Y.T.D. designed the experiments. Y.J.Z. and Y.T.D. conducted the experiments. D.L., H.Z.W., X.Z., T.T.L., J.B.W. and Y.L. contributed in pepper germplasm propagation. L.J.O., F.L. and X.X.Z. contributed part of pepper accessions. Y.J.Z. Y.T.D., B. O. and F.L. analysed the data and wrote the manuscript.

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