Review

Applications of virus-induced gene silencing for identification of gene function in fruit

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Abstract

With the development of bioinformatics, it is easy to obtain information and data about thousands of genes, but the determination of the functions of these genes depends on methods for rapid and effective functional identification. Virus-induced gene silencing (VIGS) is a mature method of gene functional identification developed over the last 20 years, which has been widely used in many research fields involving many species. Fruit quality formation is a complex biological process, which is closely related to ripening. Here, we review the progress and contribution of VIGS to our understanding of fruit biology and its advantages and disadvantages in determining gene function.

Keywords: Gene function; virus-induced gene silencing; fruit; viral vector.

Introduction

Fruit not only provides seeds for plant reproduction, but also provides an abundant source of dietary nutrients for humans (Giovannoni, 2004; Klee and Giovannoni, 2011). The formation of fruit quality attributes, such as color, flavor, texture and nutrition, is controlled by ripening-related genes during the fruit ripening process (Giovannoni et al., 2017; Li et al., 2021). The study of the molecular mechanism of fruit ripening and quality formation can provide a theoretical basis for improving fruit quality through genetic selection and transgenic breeding in the future. With the development of high-throughput sequencing technology, it has become very easy to obtain genetic information, but the identification of gene function still depends on effective methods for functional evaluation.

When double-stranded RNA (dsRNA) appears in an organism, it will induce the organism’s defense mechanisms, including a response called post transcriptional gene silencing (PTGS; Zhang et al., 2015). The PTGS uses the Dicer enzyme to cut dsRNA into small interfering RNA (siRNA). This siRNA specifically recognizes and degrades any complementary mRNA sequence in the cytoplasm. The degradation of mRNA leads to the loss of the function of the gene, by RNA interference (RNAi; Filipowicz et al., 2005). In the RNAi process, dsRNA is the inducer of RNAi, and if we can effectively introduce dsRNA of the target gene into a plant, that target mRNA in plant will effectively be silenced at the RNA level. In order to form dsRNA in plants, it is necessary to construct a vector for expression of a DNA sequence with an antisense or hairpin fragment of the target gene. This recombinant vector can be introduced into plant tissue and transgenic plants can be obtained through tissue culture so that the reduction in target gene expression is stably inherited. Expression of the transgene from the vector will induce the RNAi of the target gene in the plant, but the transgenic process required to achieve this is complex and time-consuming and is also difficult or impossible for some plant species. However, dsRNA can also be produced during virus replication. If the target gene fragment is inserted into a modified virus that is then used to infect a plant, a large number of dsRNA molecules corresponding to the target gene can be produced rapidly in the infected plant as a consequence of the virus replication, thus effectively inducing RNAi in plants using the virus as a transient gene expression vector (Pandey et al., 2015). Compared with transgenic plant, virus-induced gene silencing (VIGS) technology is simple, fast and efficient, and it is not necessary to obtain transgenic
plants through tissue culture (Burch-Smith et al., 2004). In this strategy, VIGS uses the recombinant virus to induce the organism to initiate the PTGS process during the process of infection. The characteristics of VIGS result in an RNAi process against any selected target gene added to the recombinant vector. In recent years, the fruit research field has established the application of VIGS technology in multiple species of fruit, but there are also some problems. Here, we will briefly review the progress of VIGS in this field.

Strategic Considerations for Fruit VIGS

Selection of virus vector

There are many kinds of plant viruses in nature, which can in theory be modified into suitable VIGS vectors, but only a few have actually been used for VIGS because it is hard to modify a wild virus into a VIGS vector. General plant virus vectors include tobacco mosaic virus (TMV), potato virus-X (PVX), barley stripe mosaic virus (BSMV), bean pod mottle virus (BPMV), tomato golden mosaic virus (TGMV), satellite virus (SV), cabbage leaf curl virus (CbLCV), apple latent spherical virus (ALSV), prunus necrotic ringspot virus (PNRSV), cucumber mosaic virus (CMV), citrus tristeza virus (CTV), cucumber green mottle mosaic virus (CGMMV) and pea early browning virus (PEBV) vectors, of which TRV, ALSV, CMV, PVX and CGMMV vectors have been successfully applied to mediate gene silencing in fruits (Burch-Smith et al., 2004; Zhang and Ghabrial, 2006; Lin et al., 2008; Senthil-Kumar and Mysore, 2011; Cui and Wang, 2017; Killiny, 2020; Liu et al., 2020).

Ideally, suitable VIGS vectors should not only produce very obvious virus symptoms after they infect plants but also be able to interfere with the observed phenotype caused by silencing of the target gene and could also affect plant growth. At present, the TRV vector is the most widely used, mainly because its structure is simple, it has more than 400 host species, and it can effectively infect the meristems of plants. Furthermore, the virus symptoms are mild, which will not interfere with the normal growth of plants (Bachan and Dinesh-Kumar, 2012). The process of VIGS based on TRV is shown in Figure 1.

Selection of reporter gene

A reporter gene is a marker that ensures the virus infection is effective and enables subsequent identification of plants with the VIGS construct. When we want to test whether a VIGS vector can induce gene silencing in a new plant, it is necessary to make sure that the modified virus can infect, spread and be active in the target host plant. If the virus cannot infect the plant, the reporter gene will not be silenced and cannot produce the desired phenotype. If the target plant is a host of the virus, then we can observe the silencing phenotype of a reporter gene. Phytoene desaturase (PDS) gene is a common and effective reporter gene expressed routinely in the leaves of green plants. If there is virus in the newly growing leaves, there will be virus in the newly growing leaves. If it can be detected, this indicates that the virus can infect, replicate and move in the plant. For any plant VIGS system that has never been successfully reported before, it is essential to ascertain whether a reporter gene silencing phenotype can be observed to prove the virus has infected plants. If PDS is selected as the reporter gene, a new leaf will show yellow or white patches of photobleaching in the infected plant. Real-time quantitative PCR (RT-qPCR) can then be used to detect the silencing level of the selected target gene.

Verification and analysis of gene silencing

There are two distinct aspects of VIGS that have to be evaluated in order to judge success. First, does the selected virus actually infect the target plant? This is generally evaluated by measuring whether there is virus in the newly growing leaves. If it can be detected, this indicates that the virus can infect, replicate and move in the plant. For any plant VIGS system that has never been successfully reported before, it is essential to ascertain whether a reporter gene silencing phenotype can be observed to prove the virus has infected plants. If PDS is selected as the reporter gene, a new leaf will show yellow or white patches of photobleaching in the infected plant. Real-time quantitative PCR (RT-qPCR) can then be used to detect the silencing level of the selected target gene. The silencing of fruit ripening-related genes often affects the ripening process of the fruit, and often results in different colored regions of the same fruit. If there is no color change expected in the fruit when the target gene is silenced, it is necessary to select a visualization system to identify silenced parts of the fruit for sampling. For example, if the transgenic purple tomato carrying Del and Ros1 genes from Antirrhinum majus are used, the selected target gene and Del or Ros1 gene (Orzaez et al., 2009) are co-silenced in Del and Ros1
overexpression transgenic tomato plant and it is easy to distinguish the VIGS-silenced tissue in which the target gene is silenced because it lacks the purple color. Transgenic GFP tomato is also a good visualization material (Quadrana et al., 2011), as silencing can be tested by looking for lack of fluorescence. The identified silenced tissue samples need to be further verified by RT-qPCR and then used for subsequent analysis, such as RNA-seq, proteome and metabolome, etc.

Figure 1. Brief process of VIGS based on TRV (Fu et al., 2005; Wang et al., 2016; Gao et al., 2018; Meng et al., 2018). First, the RNA1 and RNA2 of TRV are reverse-transcribed into double-stranded DNA, then inserted into a plant expression vector forming pTRV1 and pTRV2, respectively. The cDNA fragment of a candidate gene is inserted into pTRV2. pTRV1 and pTRV2-target gene fragments are then transferred to Agrobacterium before infecting plants. In the preparation for infection, Agrobacterium infiltration suspensions carrying pTRV1 and pTRV2-target gene fragments are mixed in a 1:1 ratio and used to infect plants by either leaf injection, high-pressure spray gun, vacuum infiltration, stem injection or carpopodium injection, after which the fruit-silencing phenotype is observed after a suitable time interval has passed. Screening and selection of VIGS-affected tissue is by reporter gene selection (color change or GFP fluorescence as described in the text) followed by polymerase chair reaction verification of target gene silencing and analysis of the resulting fruit phenotype. VIGS, virus-induced gene silencing; TRV, tobacco rattle virus; PDS, phytoene desaturase; GFP, green fluorescent protein.
Application of VIGS in Functional Gene Identification in Fruit

VIGS has been widely used as a rapid gene function verification method in fruits of many species (Table 1). Tomato is the most widely used fruit and TRV is the most popular virus vector. There also have been reports of successful application of VIGS in several other fruits in which it is hard to obtain transgenic plants, such as apple, peach, pear, litchi, mango, and others. In above cases, detached fruit are mostly injected with the VIGS vector, without any report of its application in a plant VIGS system to make sure that the virus can infect the plant. Therefore, it is recommended that verification is required as to whether VIGS can be effectively implemented in these kinds of fruits.

There are several examples of the functional identification of genes by VIGS in fruit. First, VIGS is often used to verify the role of a selected candidate gene related to fruit ripening or pigment regulation. Compared with the control, the fruit silenced by the candidate gene may show an uneven ripening phenotype or color change if the candidate gene plays an important role in the fruit ripening and color formation. If the candidate gene is a negative regulator, the silenced part of the fruit will ripen prior to the non-silenced fruit part. In the case of the silencing of a positive regulator, one aspect of ripening, such as color change involving the accumulation or degradation of chlorophyll, anthocyanin and lycopene metabolism will be affected and it is easy to show a visible phenotype, which can be used to verify the function of candidate genes. VIGS can also be used to screen transcription factors family members when one member has been shown to be involved in fruit ripening, such as the NAC transcription factors (TFs) family. Gao et al. (2018) used VIGS to screen 34 NAC (NAM, ATAF1, ATAF2 and CUC2) TF family members and found that non-ripening-like1 (SNOR-like1) is a positive regulator of fruit ripening. VIGS of SNOR-like1 produced an uneven fruit ripening phenotype, and RT-qPCR detection of delayed ripening sites showed that the expression level of SNOR-like1 was significantly reduced. VIGS was also used to screen seven HD-Zip family members with high expression levels in fruit and found that BEL1-LIKE HOMEODOMAIN 11 (SIBEL11) negatively regulated the synthesis of chlorophyll in tomato fruit and expression of SIBEL11 was significantly inhibited in the region where chlorophyll accumulation was accelerated (Meng et al., 2018). The HD-Zip homeobox protein 1 (LeHb-1) gene was silenced by PVX to show delayed ripening in tomato fruit (Lin et al., 2008). VIGS can be used to screen the function of ripening-related proteins. Wang et al. (2014) used VIGS to investigate the functions of different proteins obtained from proteomics studies and found that two E2s genes, ubiquitin-conjugating enzymes 32 (SIUBC32) and ubiquitin-conjugating enzymes 41 (SIUBC41), are involved in the regulation of fruit ripening. The infected fruit again showed an uneven ripening phenotype during the ripening process, with delayed yellow regions compared with unaffected orange regions, and the expression levels of SIUBC32 and SIUBC41 in the yellow region with delayed ripening were significantly decreased. Zhu et al. (2015) used VIGS to screen long non-coding RNAs (lncRNAs) with high expression level during fruit ripening and found that lncRNA1459 and lncRNA1840 regulate tomato fruit ripening, and the ripening process of the silenced parts of lncRNA1459 and lncRNA1840 VIGS fruit was significantly delayed.

An important feature of the VIGS approach is that it is possible to screen more than 100 candidate genes in tomato fruit in a short time. This can be done by sprout vacuum-infiltration (Yan et al., 2012). For example, if it is desired to screen the genes related to fruit ripening from 100 candidate genes by VIGS, a fast and high throughput operation can be used, as follows. Firstly, 300–500 bp fragments of these 100 candidate genes are cloned and inserted into the TRV vector by the In-fusion method to obtain 100 candidate gene TRV–candidate gene vectors numbered from 1 to 100, and transformed into Agrobacterium tumefaciens GV3101 for the next step. Secondly, prepare 1000 sprouts of Micro-Tom tomato. Place each group of 10 sprouts into 2-mL centrifuge tubes numbered from 1 to 100. Add to each centrifuge tube 1 mL Agrobacterium tumefaciens infection solution with the same number. Then, place the 100 centrifuge tubes in a tube rack fitted with an opening cover for use during vacuum infiltration. Subsequently, all infected sprouts are transferred into soil and cultured under appropriate temperature and humidity conditions. After one month the seedlings grow, blossom and bear fruit. The color change of fruits during ripening can be observed and the candidate genes corresponding to fruits with uneven ripening phenotype selected for subsequent identification. At the same time, 10 sprouts infected by TRV-PDS can be used as control to evaluate the effectiveness and efficiency of the infection process of the same batch. This process takes about 3e months to screen 100 candidate genes (Figure 2).

Conclusions

VIGS can play an important role in the field of fruit ripening research. The use of TRV vectors is a mature technology and has been successfully applied to good effect in fruit function research. The feasibility and effectiveness of TRV in pear, peach, apple and other fruits still needs further study, because no reference has demonstrated that TRV can cause effective reporter gene silencing, for example by PDS silencing in the seedlings. Apart from TRV vectors, few other effective vectors have been reported. It is necessary to develop easy-to-use VIGS vectors with mild viral symptoms that are suitable for use with a wide range of hosts. It is also necessary to explore and establish a variety of effective visualization methods for use with the VIGS technology system, in order to identify silenced tissue, because it is hard to silence all fruit tissue completely. At present, fruit VIGS is mostly used in fruit ripening, but it could be widely used in fruit nutrition metabolism and disease resistance research in the future. In addition to its application in the identification of fruit gene function, VIGS could also be applied in the study of gene functions in various aspects of plant biology, such as disease resistance (Peart et al., 2002; Wei et al., 2018; Situ et al., 2020), stress response (Zhang J. X. et al., 2018, Zhang G. F. et al., 2020) and organ development (Chen et al., 2021). Compared with other transgenic technologies based on tissue culture, such as CRISPR/Cas9 technology, RNAi technology and strong promoter-based overexpression technology, VIGS technology has the advantages of simple and rapid operation (Burch-Smith et al., 2004). It can also be used for efficient high-throughput screening and functional verification of candidate genes without involving a cumbersome tissue culture process. However, due to the fact that gene silencing mediated by VIGS technology in plants cannot be intergenerational and complete silencing of target genes cannot be achieved, further studies on plant gene function still mainly rely on obtaining transgenic plants that stably inherit the modification through tissue culture.
Table 1. Applications of VIGS in functional gene identification of fruit

| Function                                      | Target gene | Virus | Fruit species | Reference                          |
|------------------------------------------------|-------------|-------|---------------|------------------------------------|
| Disease resistance                            | MdCNGC2     | TRV   | Apple         | Zhou et al. (2020)                 |
| Anthocyanin biosynthesis                       | MdHB1       | TRV   | Apple         | Jiang et al. (2017)                |
| Fruit ripening                                | MdERF2      | TRV   | Apple         | Li T. et al. (2016)                |
| Fruit ripening                                | FvTCP9      | TRV   | Strawberry    | Xie et al. (2020)                  |
| Fruit ripening                                | FaIPK1      | TRV   | Strawberry    | Hou et al. (2018)                  |
| Metabolism of proanthocyanidins                | FaMYB5      | TRV   | Strawberry    | Wang L. et al. (2017)              |
| Fruit ripening                                | FaABI4      | TRV   | Strawberry    | Chai and Shen (2016)               |
| Fruit coloration                               | PpGST1      | TRV   | Peach         | Zhao Y. et al. (2019)              |
| Fruit softening                                | PpBGAL10, PpBGAL16 | TRV   | Peach         | Liu H. K. et al. (2018)            |
| Carotenoid degradation                         | PrepeSEP1   | TRV   | Peach         | Li et al. (2017)                   |
| Carotenoid biosynthesis                        | CDD4        | TRV   | Peach         | Bai et al. (2016)                  |
| Fruit ripening                                | PpCHLH      | TRV   | Peach         | Jia et al. (2011)                  |
| Color formation and carotenoid accumulation    | ZEP         | TRV   | Pepper        | Lee et al. (2021)                  |
| Carotenoid metabolism                          | CaPSY1      | TRV   | Pepper        | Wei et al. (2021)                  |
| Color formation                                | PSY2        | TRV   | Pepper        | Jang et al. (2020)                 |
| Carotenoid accumulation                        | PRR2        | TRV   | Pepper        | Jeong et al. (2020)                |
| Fruit ripening                                | CaMET1-like1| TRV   | Pepper        | Xiao et al. (2020)                 |
| Capsaicin biosynthesis                         | pAMT        | TRV   | ASW           | Li C. J. et al. (2019)             |
| Anthocyanin biosynthesis                       | An2         | TRV   | Pepper        | Kim et al. (2017)                  |
| Capsaicinoid accumulation                      | Tpm1        | CMV   | Pepper        | Ogawa et al. (2015)                |
| Capsanthin synthesis                           | Cca, Ppy, Lcyb, Crtz | TRV   | Pepper        | Tian et al. (2014)                 |
| Piperine biosynthesis                          | PipCoA ligase| TRV   | Black pepper | Schnabel et al. (2020)             |
| Climacteric response                           | SlICDH1     | TRV   | Tomato        | Gamrasni et al. (2020)             |
| Fruit ripening                                | Sir1P1b     | TRV   | Tomato        | Yang et al. (2020)                 |
| Fruit quality                                  | SITDR4      | TRV   | Tomato        | Zhao X. D. et al. (2019)           |
| Fruit ripening                                | SPtPS       | TRV   | Tomato        | Naing et al. (2019)                |
| Fruit ripening                                | LncRNA2155  | TRV   | Tomato        | Yu T. T. et al. (2019)             |
| Chloroplast development and chlorophyll synthesis| SibeL11    | TRV   | Tomato        | Meng et al. (2018)                 |
| Fruit ripening                                | SinoR-like1 | TRV   | Tomato        | Gao et al. (2018)                  |
| Fruit ripening                                | SdORM4      | TRV   | Tomato        | Yang et al. (2017)                 |
| Lycopene accumulation                          | SinaP7      | TRV   | Tomato        | Fu et al. (2016)                   |
| Fruit ripening                                | LcSPL-CN    | TRV   | Tomato        | Lai et al. (2015)                  |
| Fruit ripening                                | LncRNA1459, LncRNA1840 | TRV   | Tomato        | Zhu et al. (2015)                  |
| Fruit ripening                                | SlUBC32, SlUBC41 | TRV   | Tomato        | Wang et al. (2014)                 |
| Ethylene biosynthesis                          | LeRIN, LeACS2, LeACS4, LeACO1 | TRV   | Tomato        | Li et al. (2011)                   |
| Fruit ripening                                | LeHb-1      | PVX   | Tomato        | Lin et al. (2008)                  |
| Ethylene response                              | LeETR4      | TRV   | Tomato        | Zhang et al. (2008)                |
| Ethylene response                              | LeCTR1, LeEL1a, LeEIN2 | TRV   | Tomato        | Fu et al. (2005)                   |
| Fruit size                                     | P5          | TRV   | Physalis      | Gao et al. (2020)                  |
| Fruit ripening and softening                   | PafME1, PafME2 | TRV   | Sweet cherry  | Qi et al. (2020)                   |
| Fruit size and ripening                        | PafCYP78A6  | TRV   | Sweet cherry  | Qi et al. (2019)                   |
| Flavonoid biosynthesis                         | ANS         | TRV   | Sweet cherry  | Qi et al. (2018)                   |
| ABA-regulated anthocyanin biosynthesis         | PucMYBA     | TRV   | Sweet cherry  | Shen et al. (2014)                 |
| Modifies epidermal cells and gravitropism      | SmCHS       | TRV   | Eggplant      | Wang and Fu (2018)                 |
| Phytoene desaturase biosynthesis               | CePD      | TRV   | Cysticapsorus vesicaria | Hidalgo et al. (2012) |
| Biosynthesis of limonoids                      | GoASC       | TRV   | Citrus         | Wang F.S. et al. (2017)            |
| Anthocyanin biosynthesis                       | VoMYBA1     | TRV   | Grape berry   | Zhang P.F. et al. (2020)           |
| Carotenoid accumulation and coloration         | CYC-B       | TRV   | Loquat         | Hong et al. (2019)                 |
| Carotenoid accumulation                        | PSY         | TRV   | Loquat         | Hong et al. (2017)                 |
| Phytoene desaturase biosynthesis               | PDS         | CGMMV | Cucurbit      | Liu et al. (2020)                  |
| Anthocyanin accumulation                       | AcMYB10     | TRV   | Red-fleshed kiwifruit | Yu M. et al. (2019)               |
| Anthocyanin biosynthesis                       | AcUFGT3a    | TRV   | Red-fleshed kiwifruit | Liu Y. F. et al. (2018)            |
| Anthocyanin accumulation                       | PpBBX16     | TRV   | Pear          | Bai et al. (2019)                  |
| Betalain biosynthesis                          | HmnWRKY40   | TRV   | Pitaya        | Zhang et al. (2021)                |
| Brassinosteroid biosynthesis                   | HrCYP90B1   | TRV   | Sea buckthorn | Liu et al. (2021)                  |
| Anthocyanin accumulation                       | MmCHI2      | TRV   | Mulberry      | Ciao et al. (2021)                 |
| Anthocyanin biosynthesis and fruit coloration  | LbNCE1D1    | TRV   | Lycium        | Li G. et al. (2019)                |
Table 1. Continued

| Function                          | Target gene | Virus      | Fruit species | Reference                  |
|-----------------------------------|-------------|------------|---------------|----------------------------|
| Early development of fruits and seeds | XsERS       | TRV        | Xanthoceras sorbifolium | Zhou and Cai (2021)         |
| Fruit ripening and pericarp coloration | RCCR        | TRV        | Mango         | Liu K. L. et al. (2018)    |
| Seed development                  | LcCWIN2, LcCWIN5 | TRV        | Litchi        | Zhang J. Q. et al. (2018)  |
| Color formation                    | LcUFGT1     | TRV        | Litchi        | Li X. J. et al. (2016)     |

CNGC2, cyclic nucleotide-gated ion channel 2; ERF2, ethylene response factor 2; TCP9, teosinte branched 1, cycloidea, and proliferating4 cell factor 9; RIPK1, red-inalual protein kinase 1; MYB5, v-myb avian myeloblastosis viral oncogene homolog 5; ABI4, ABI insensitive 4; GST1, glutathione S-transferase 1; BGAL10, beta-galactosidase 10; BGAL16, beta-galactosidase 16; SEP1, sepalata 1; CCD4, carotenoid cleavage dioxygenase 4; CHLH, magnesium chelatase H subunit; ZEP, zeaxanthin epoxidase; PSY1, phytoene synthase 1; PSY2, phytoene synthase 2; PRR2, pseudo response regulator2; MET1-like1, methyltransferase 1-like1; pAMT, putative aminotransferase, An2, Anthocyanin2; Pun1, Punnet gene 1; Cca, capsanthin/capsorubin synthase; Lcyb, lycopene-beta-cyclase; Crtz, beta-carotene hydroxylase; PipCoA ligase, piperic acid coenzyme A ligase; ICDH1, isocitrate dehydrogenase 1; RIP1b, RNA editing factor interacting protein 1b; TDR4, FRUITFULL 1; ORRM4, organelle RNA recognition motif-containing protein 4; NAP7, non-intrinsic ARC protein 7; SPL-CNR, squamosa promoter-binding-like-colorless non-ripening; RIN, ripening inhibitor; ACS2, 1-amincyclopropane-1-carboxylate synthase 2; ACS4, 1-amincyclopropane-1-carboxylate synthase 4; ACO1, 1-amincyclopropane-1-carboxylate oxidase 1; ETR4, ethylene receptor 4; EILs, ethylene insensitive 3-like protein; P5, physalis lateral organ boundaries domain family transcription factor; PME1, pectin methyl esterase 1; PME2, pectin methyl esterase 2; CYP78A6, cytochrome P450, family 90, subfamily A, polypeptide 6; ANS, anthocyanidin synthase; MYBA, v-myb avian myeloblastosis viral oncogene homolog A; CHS, chalcone synthase; OSC, oxidosqualene cyclase; MYB1, v-myb avian myeloblastosis viral oncogene homolog A1; CYC-B, chromoplast-specific lycopene beta-cyclase; MYB10, v-myb avian myeloblastosis viral oncogene homolog 10; UFGT3a, uridine diphosphate flavonoid glycosyltransferase 3a; BBX16, B-box domain protein 16; WRKY40, WRKY DNA-binding protein 40; CYP90B1, cytochrome P450, family 90, subfamily B, polypeptide 1; CHI2, chalcone isomerase 2; NCED1, 9-cis-epoxycarotenoid dioxygenase 1; ERS, ethylene receptor homolog; RCCR, red chlorophyll catabolite reductase; CWIN2, cell wall invertase 2; CWIN3, cell wall invertase 3; UFGT1, uridine diphosphate flavonoid glycosyltransferase 1; TRV, tobacco rattle virus.

Figure 2. High-throughput screening of VIGS based on sprout vacuum-infiltration (Yan et al., 2012). First, sufficient seeds of tomato (cv. Micro-Tom) are germinated in water at 28 °C for 2-3 days to reach a length of 0.5-1 cm. At the same time, the Agrobacterium infection solutions carrying pTRV1 or pTRV2-candidate gene fragments are prepared and mixed in a 1:1 ratio. Then 10 seeds and 1 mL mixed infection solution are added to a 2-mL centrifuge tube with a cover that can be opened for vacuum-infiltration. Infected sprouts are planted in commercially available vegetative soil, and the silencing phenotype of fruit can be observed as the plants develop. For example, PDS-silenced fruit will show a phenotype of photobleaching. VIGS, virus-induced gene silencing; TRV, tobacco rattle virus; PDS, phytoene desaturase.
Author Contributions
Gangshuai Lu: Writing original draft, review and editing. Hongli Li: Writing, review and editing. Daqi Fu: Design, writing original draft, review and editing, supervision.

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Conflict of Interest
The authors declare no conflict of interest.

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Gangshuai Lu: Writing original draft, review and editing. Hongli Li: Writing, review and editing. Daqi Fu: Design, writing original draft, review and editing, supervision.

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