Horticultural innovation by viral-induced gene regulation of carotenogenesis

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Abstract

Multipartite viral vectors provide a simple, inexpensive and effective biotechnological tool to transiently manipulate (i.e. reduce or increase) gene expression in planta and characterise the function of genetic traits. The development of virus-induced gene regulation (VIGR) systems usually involve the targeted silencing or overexpression of genes involved in pigment biosynthesis or degradation in plastids, thereby providing rapid visual assessment of success in establishing RNA- or DNA-based VIGR systems in planta. Carotenoids, pigments provide plant tissues with an array of yellow, orange, and pinkish-red colours. VIGR-induced transient manipulation of carotenoid-related gene expression has advanced our understanding of carotenoid biosynthesis, regulation, accumulation and degradation, as well as plastid signalling processes. In this review, we describe mechanisms of VIGR, the importance of carotenoids as visual markers of technology development, and knowledge gained through manipulating carotenogenesis in model plants as well as horticultural crops not always amenable to transgenic approaches. We outline how VIGR can be utilised in plants to fast-track the characterisation of gene function(s), accelerate fruit tree breeding programs, edit genomes, and biofortify plant products enriched in carotenoid micronutrients for horticultural innovation.

Introduction to carotenoid functional genomics mediated by virus-induced gene regulation (VIGR)

There is an ongoing arms race between host plants and their infectious viruses. Viruses have evolved virulence factors which can hijack the plant’s immune system to enable effective viral infection and replication. In response, host plants have co-evolved sequence-specific RNA-mediated viral gene silencing mechanisms that recognise and destroy the double-stranded RNA (dsRNA) replication intermediate [1]. Researchers have leveraged this mechanism of viral infection and host-mediated RNA defense to fast-track basic research, biotechnology, and plant breeding ventures. That is, a partial mRNA sequence of interest can be introduced into a modified viral vector and upon host infection causes RNA-mediated gene silencing of the target gene in planta. A widely established visual reporter system used to engineer viral vector systems has involved silencing genes involved in carotenoid or pigment biosynthesis. For example, the tobacco mosaic virus-based virus-induced gene silencing (VIGS) system was first reported in 1995 to silence carotenoid pigment biosynthesis in Nicotiana benthamiana leaves, that caused white or bleached sectors to appear due to an inhibition of chloroplast biogenesis [2]. Carotenoids are a diverse subgroup of isoprenoid secondary metabolites synthesized in eukaryotic plants, algae and fungi, as well as some prokaryotic cyanobacteria and bacteria [3]. In plants, carotenoids are synthesized within cellular plastids and provide different shades of colour, between yellow and pinkish red, to various plant parts including tubers, leaves, flowers, fruits, and seeds. The colour of carotenoids is dependent on the desaturation of their 40-carbon linear backbone that is conjugated with double bonds [4]. The increase in desaturation, from 3 to a maximum of 15 bonds, shifts the colour spectrum from yellow to orange to red. Carotenoids provide essential functions in plants, they assist to capture light photons required for photosynthesis, protect cells from photooxidation and scavenge reactive oxygen species [5]. They also provide precursors for the biosynthesis of phytohormones and apocarotenoid signalling molecules that maintain plastid development, cellular homeostasis, regulate growth and development, attract mycorrhiza or insect pollinators, and deter insect feeding. Several comprehensive reviews have been published describing...
carotenoid biosynthesis, regulation, degradation, and storage, as well as their functions [3, 6–12].

The genes required for carotenoid metabolism were initially functionally characterised using traditional methods (e.g. transgenic overexpression or silencing, and mutant screening). However, VIGR has become a powerful transient tool to study carotenogenesis in non-model horticultural crops such as pepper, cassava, California poppy, peaches, loquat, tomato and strawberry where transgenic approaches can be time consuming to functionally characterise multiple alleles of a carotenoid associated gene (Fig. 1). In this review, we briefly discuss the mechanisms of VIGR (silencing and overexpression), highlight developments in VIGR technology using carotenoid pigmentation as a visual marker of success, and reveal how VIGR has been used to manipulate carotenoid biosynthesis, storage, degradation, regulation, and plastid signalling in planta. We discuss potential applications of VIGR to advance horticultural innovation and plant-breeding with a view towards biofortifying carotenoid enriched and more nutritional crop products.

### Molecular mechanisms of VIGR in planta
#### Virus-induced gene silencing (VIGS)

VIGS is based upon the mechanism of homology-based RNA-mediated gene silencing that involves first, cleaving of dsRNA formed during viral replication to produce small interfering RNAs (siRNAs) and, second recruiting siRNAs as a guide to cleave other homologous mRNAs (Fig. 2) [13–15]. The process of dsRNA synthesis in virus infected cells depends on the type of viral nucleic acid. For positive-sense (+) single-stranded (ss) RNA viruses, viral genomic RNA is first translated to produce different viral proteins including RNA-dependent RNA polymerases (RdRPs) and other replication factors that are used to synthesize negative-stranded RNA from the ssRNA viral parental template [16–18]. The neosynthesized negative strands serve as a template for synthesis of the positive strand RNA. The dsRNA can be formed either by complete or partial annealing of positive and negative RNA strands, or by folding of positive RNA strands to develop secondary stem-loop-like structures. In ssDNA viruses, such as geminiviruses, the parental single-stranded viral DNA is used as a template to directly synthesize dsDNA that occurs in the nucleus through a rolling-cycle mechanism [19]. The dsDNA is then bidirectionally transcribed to produce positive- and negative-stranded polycistronic RNAs which anneal to form dsRNA [20, 21] (Fig. 2).

Plants have multiple homologs of the host-encoded DICER-like (DCL) endoribonucleases that recognise dsRNA and cleave it to synthesize 21–24 nucleotide (nt) long primary siRNAs [22]. In Arabidopsis there are four DCL genes present [23]. DCL4 is responsible for production of 21 nt siRNAs, the most abundant type of siRNA synthesized during RNA virus-induced gene silencing [24]. DCL2 and DCL3 are more actively involved in producing 22 nt and 24 nt siRNAs, respectively. DCL1 produces microRNAs; however it can produce siRNAs when a host becomes infected with geminivirus or caulimovirus DNA viruses [25]. DCL1 can also become more active in processing siRNAs when other DCLs are absent [25]. The neofunctionalization and redundancy of different DCLs indicate that plants have evolved their preparedness to strengthen RNA-mediated defense mechanisms against viral infection using siRNAs generated from perfect and imperfect dsRNA even when one or more DCL homologs may not be functional. As an example, TRV-mediated VIGS of PHYTOENE DESATURASE (PDS) using a 21 nucleotide guide from N. benthamiana worked successfully in a wide range of Solanaceous plant species, and heterologous sequences from distant plant species silenced the PDS ortholog in N. benthamiana demonstrating the effectiveness of the siRNA system to mount defense through gene silencing [26].

The siRNAs help guide, maintain, and spread post-transcriptional gene silencing processes throughout plant tissues. During silencing, the guide strand of siRNA is loaded into an ARGONAUTE family protein, AGO1, to form a multicomponent RNA-induced silencing complex (RISC) [27]. The guide siRNA strand provides specificity in the RISC complex to target viral RNAs, endogenous mRNAs, or heterologous RNA [28]. The primary siRNAs generated through cleaving of viral dsRNA are capable of inducing silencing of the targeted gene of interest (GOI) in hosts, however, studies indicate that secondary siRNAs (formed by cleavage of dsRNA generated by host RDRP using primary siRNA as a template) enhance the maintenance and spread of VIGS [29–32] (Fig. 2). The spread of siRNA can occur locally through plasmodesmata and systemically via the phloem to silence expression of the GOI in uninfected tissues [30, 33, 34]. Although, silencing does not always proceed in a systemic manner as exemplified when California poppy plants were inoculated with a TRV-based VIGS vector targeting PDS, the white sectors resulting from the inhibition of carotenoid biosynthesis were restricted to certain organs or parts of organs [35].

The VIGS system has also been used to interrogate heritable transcriptional gene silencing (TGS) through RNA directed DNA methylation [36]. VIGS-induced TGS becomes inhibited at higher temperatures, reducing unwanted transmission of epigenetic gene silencing to subsequent generations, which can facilitate TGS-free crop improvement [37]. Details regarding the biogenesis and environmental regulation of plant small RNAs, as well as diversification of silencing pathways through the expansion of RNA-dependent RNA polymerases, DICER proteins and ARGONAUTE proteins have been reviewed elsewhere [38].

#### Virus-induced gene overexpression (VIGO)

Viral vectors can also serve to transiently overexpress a foreign or endogenous GOI in plants. In contrast to viral vectors that harbor a partial sequence of a GOI
that triggers RNA-mediated silencing of targeted gene expression, VIGO vectors contain a full-length coding sequence (CDS) of the GOI inserted in-frame within the viral coding region (Fig. 2) [39]. The CDS of the GOI is translated together with the viral genome during virus replication in infected cells resulting in a high copy number of synthesised proteins. The wild-type virus usually has a limited cargo capacity to accommodate a foreign GOI [39–41]. Elimination of viral coding sequences that are not necessary for virus replication in the host, such

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as coat protein or genes required for virus transmission via insect/nematodes, can help to increase the capacity to overexpress larger GOI in the VIGO vector system [42]. For example, deletion of the NUCLEAR INCLUSION b (Nib) gene from potyvirus plus-strand RNA based vector system of Tobacco etch virus (TEV) increased capacity to allow introduction of a carotenoid gene cluster (e.g. GERANYLGERANYL DIPHOSPHATE SYNTHASE I; crtE, PHYTOENE SYNTHASE; crtB, and PHYTOENE DESATURASE; crtI) from Pantoea ananatis, that was flanked by native cleavage sites to allow transient processing of these proteins [43] (Fig. 1). This strategy enabled the
heterologous manipulation of the carotenoid pathway to produce the health-promoting carotenoid lycopene in tobacco leaf tissues transformed with the \textit{Nib} transgene [44]. This highlights an engineering challenge when eliminating regions important for viral movement and/or replication, in that the altered virus may become restricted in its ability to systemically spread and/or produce sufficient protein levels within host tissues.

**Viruses and vectors for VIGR**

The engineering of viral vectors to transiently regulate gene expression has advanced rapidly in recent years providing a suite of over fifty viral expression systems, mostly developed from +ssRNA viruses, DNA viruses as well as negative-sense RNA viruses [45, 46]. Tobacco rattle virus (TRV) has been engineered to become the “gold-standard” bipartite (genome divided into two segments)+ ssRNA viral vector commonly used for VIGS. TRV-based VIGS expression systems have been successfully established in a wide range of hosts including many horticultural crops such as: tomato (\textit{Solanum lycopersicum}), chili pepper (\textit{Capsicum annuum}), strawberry (\textit{Fragaria ananassa}), peach (\textit{Prunus persica}), California poppy (\textit{Eschscholzia californica}), Jatropha curcas, petunia (\textit{Petunia hybrida}), Four O’Clock (\textit{Mirabilis jalapa}), loquat (\textit{Eriobotrya japonica}) and rose (\textit{Rosa hybrida}) [35, 47–55]. The Apple latent spherical virus, \textit{Prunus necrotic ringspot virus}, \textit{Citrus tristeza virus} and \textit{Citrus leaf blotch virus} are +ssRNA viruses that have also been used to regulate gene expression in fruit trees including apple, peach and citrus [56–60].

DNA viruses from the family \textit{Geminiviridae}, which have a single-stranded circular genome, have also been developed for VIGS. For example, the East African cassava mosaic virus, \textit{Pepper huasteco yellow vein virus}, \textit{Tomato yellow leaf curl China virus}, \textit{Beet curly top virus}, Tobacco curly shoot virus and \textit{Tomato leaf curl virus} have been successfully used to silence gene expression in cassava (\textit{Manihot esculenta}), tomato (\textit{S. lycopersicum}), \textit{C. annuum}, spinach (\textit{Spinacia oleracea}) or \textit{P. hybrida} [61–67]. Despite the development of effective VIGS expression systems for over 130 plant species to date, there are still elusive horticultural tree species such as mango, macadamia and avocado that are not yet amenable to VIGR. An intriguing aspect is that at least 43 reports of VIGR describe the manipulation of carotenoid metabolism (Table 1). Carotenoid pigments provide a favourable visual marker of VIGR success, and for the remainder of this review we focus on how VIGR has advanced our knowledge of carotenoid biosynthesis and metabolism in plants.

**Insights into carotenoid biosynthesis and regulation by VIGR**

VIGR has served as an important tool to enhance our knowledge regarding carotenoid biosynthesis and regulatory pathways in plants. Instances include when; 1) transgenic approaches are not always possible, time- and/or cost-effective in non-model species [35], 2) combinatorial silencing of multiple pathway targets or simultaneous overexpression of several genes is necessary [44], 3) the loss-of-function of the carotenoid gene via mutation or transgenic approaches causes lethality making it difficult to study tissue specific gene functions (e.g. ZDS [120], and 4) there are multiple homologs, alleles and/or redundancies in gene functions (e.g CCD) [99].

The first committed step in carotenoid biosynthesis involves the catalytic conversion of all-trans geranylgeranyl diphosphate (GGPP) into 15-cis phytoene in plastids by \textit{PHYTOENE SYNTHASE (PSY)} [121] (Fig. 1). Formation of 15-cis phytoene from GGPP is a rate limiting step in carotenoid biosynthesis and perturbation in this step can significantly reduce downstream product accumulation. PSY downregulation impairs plastid development, pigmentation and colour thereby providing a reporter system to develop VIGR systems [95]. For example, silencing of PSY in tomato fruits with a TRV-based VIGS expression system (herein silencing of any gene/mRNA will refer to silencing using TRV-based VIGS expression system unless otherwise stated) resulted in 90% silencing of PSY mRNA and a 30-fold reduction in total carotenoid content [109]. Fruits with silenced PSY were completely devoid of cis-carotenones and had significantly lower levels of lutein and \(\beta\)-carotene. Silencing PSY in detached pepper and loquat fruits also caused a significant reduction in PSY transcript levels and carotenoid content [75, 83]. Similarly, silencing of PSY in green leaves of California poppy and cassava led to a whitish/bleached appearance in leaves, indicative of a drastic decrease in carotenoids and chlorophyll pigmentation due to a retrograde impairment in chloroplast biogenesis [84, 95].

VIGS systems have proven utility to advance functional genomics studies by rapidly enabling the dissection of functional redundancy and tissue specific expression patterns of homologous genes through the simultaneous silencing of gene alleles (targeting conserved sequences) or silencing of an individual gene (targeting unique regions) within a gene family. Many horticultural plants including tomato, apple, loquat, citrus, maize, rice and \textit{Brassica} spp. have multiple homologs of PSY that are functionally diverse and transcribed in a tissue-specific manner [122–126]. In tomato, PSY1 is highly expressed in fruits during the breaker stage and fruit ripening, whereas other homologs \textit{PSY2} and \textit{PSY3} are mostly expressed in chloroplast-containing tissues and roots, respectively [109, 127]. Silencing of \textit{PSY1} at the breaker-stage during fruit development resulted in a reduction in carotenoids and development of a yellow coloured fruit phenotype similar to that of the natural yellow-flesh \textit{psy1} mutant in tomato accession “\textit{yellow flesh}” [35,32] [109]. Unlike \textit{PSY1}, silencing of \textit{PSY2} did not cause a significant change in carotenoid content or composition, which supports the conclusion that \textit{PSY2} may not play a significant role during tomato fruit development [128]. In contrast, silencing of the \textit{PSY3} in tomato fruits reduced phytoene, phytofluene and \(\zeta\)-carotene, revealing
Table 1. VIGR systems utilised to manipulate gene expression governing carotenoid metabolism and plastid biogenesis in horticultural plant species. Abbreviations: VIGS: virus-induced gene silencing, ALSV: Apple latent spherical virus, BCTV: Beet curly top virus, BSMV: Barley stripe mosaic virus, CGMMV: cucumber green mottle mosaic virus, CLBV: Citrus leaf blotch virus, CLCV: Cotton leaf crumple virus, CMV: Cucumber mosaic virus, CTV: Citrus tristeza virus, DNAβ- TYLCVV: DNAβ- of Tomato yellow leaf curl China virus, EACMV: East African cassava mosaic virus, GLRaV-2: Grapevine leafroll-associated virus-2, GVA: Grapevine virus A, PEBV: Pea early brown virus, FNRR: Prunus necrotic ring spot virus, PVX: Potato Virus X, TRV: Tobacco etch virus, TEV: Tobacco rattle virus, TRSV: Tobacco ring spot virus, TYMV: Turnip yellow mosaic virus, PDS: PHYTOENE DESATURASE, PSY: PHYTOENE SYNTHASE, ZDS: ζ-CAROTENE DESATURASE, ZISO: ζ-CAROTENE ISOMERASE, CRTISO: CAROTENOID ISOMERASE, β-LYC: Lycopene β-CYCLASE, ε-LYC: Lycopene ε-CYCLASE, β-OHase: β-CAROTENE HYDROXYLASE, ZEP: ZEAXANTHIN EPOXIDASE, CCS: CAPSANTHIN/CAPSORUBIN SYNTHASE, NCED: 9-CIS-EPOXYCAROTENOID DIOXYGENASE, CCD: CAROTENOID CLEAVAGE DIOXYGENASE, CHLH: MAGNESIUM CHELATASE H-SUBUNIT, CHLI: MAGNESIUM CHELATASE I-SUBUNIT, CRTE: GERANYLGERANYL CAPSANTHIN/CAPSORUBIN SYNTHASE, TRV: TOBACCO ETCH VIRUS, TEV: TOBACCO RATTLE VIRUS, TYMV: TURNIP YELLOW MOSAIC VIRUS, WCMV: WHITE CLOVER MOSAIC VIRUS, PDS: PHYTOENE DESATURASE, PSY: PHYTOENE SYNTHASE, ZDS: ζ-CAROTENE DESATURASE, ZISO: ζ-CAROTENE ISOMERASE, CRTISO: CAROTENOID ISOMERASE, β-LYC: Lycopene β-CYCLASE, ε-LYC: Lycopene ε-CYCLASE, β-OHase: β-CAROTENE HYDROXYLASE, ZEP: ZEAXANTHIN EPOXIDASE, CCS: CAPSANTHIN/CAPSORUBIN SYNTHASE, NCED: 9-CIS-EPOXYCAROTENOID DIOXYGENASE, CCD: CAROTENOID CLEAVAGE DIOXYGENASE, CHLH: MAGNESIUM CHELATASE H-SUBUNIT, CHLI: MAGNESIUM CHELATASE I-SUBUNIT, CRTE: GERANYLGERANYL DIPHOSPHATE SYNTHASE, CRTB: PHYTOENE SYNTHASE, CRTI: PHYTOENE DESATURASE.

| Plants | Viruses used for developing VIGR system | Gene Regulated (All are silenced unless indicated) | Plant tissue | Reference |
|--------|----------------------------------------|---------------------------------------------------|--------------|-----------|
| Aquilegia vulgaris | TRV | PDS | Leaf and flower | [68] |
| Brassica campestris | TYMV | PDS | Leaf | [69] |
| Brassica nigra | TRV | PDS | Leaf | [70] |
| Brassica rapa | TYMV | PDS | Leaf, stem, flower, silique and stalk | [71] |
| Cannabis sativa | CLCV | PDS, ChlI | Leaf | [72] |
| Capsicum annuum | TRV | PDS, PDS, ε-LYC, β-OHase, ZEP, CCS | Leaf and fruit | [73, 74] |
| Catharanthus roseus (periwinkle) | TRV | PDS, ChlH | Leaf and flower | [76, 77] |
| Citrus spp. (Watermelon, melon, cucumber, bottle gourd, Zucchini, sponge gourd) | CTV, CLBV | PDS | Leaf | [58, 78] |
| Cucurbitus (Watermelon, melon, cucumber, bottle gourd, Zucchini, sponge gourd) | CGMMV, TRV, TRSV, ALSV | PDS | Leaf | [79, 80, 81, 82] |
| Enoplota japonica (Loquat) | TRV | PDS, ChlI, ChlI | Leaf and flower | [87] |
| Eucholzia californica (California poppy) | TRV | PDS, PDS, ZDS, β-OHase, ZEP | Leaf | [88] |
| Frangula x ananassa (Strawberry) | TRV, ALSV | PDS, NCED1 | Leaf and fruit | [49, 85, 86] |
| Gerbera hybrida (Gerbera) | TRV | PDS, ChlI, ChlI | Leaf and flower | [89] |
| Gladiolus hybrida | TRV | PDS, ChlI, ChlI | Leaf | [51] |
| Jalapa curcas | TRV | PDS| Leaf | [50] |
| Lactuca sativa (Lettuce) | LMV | CrtB (VIGO) | Leaf | [91] |
| Lathyrus odorata | PEBV | PDS | Leaf | [90] |
| Lilium leichtlinii (Lilies) | CMV | PDS | Leaf and flower | [91] |
| Lithichlo denisii Sonn. (Litchi) | TRV | PDS | Leaf | [92] |
| Lycium spp. (Wolfberry) | TRV | NCED1, PDS, ChlI | Leaf and fruit | [93, 94] |
| Malus domestica (Apple) | ALSV | PDS | Leaf | [56] |
| Manihot esculenta (Cassava) | EACMV | PDS | Leaf | [61, 62, 95] |
| Mimulus guttatus | TRV | PDS | Leaf | [96] |
| Mirabilis jalapa | TRV | PDS | Leaf | [53] |
| Nicotiana tabacum (Tobacco) | TRV | PDS, ε-LYC | Leaf | [97, 98] |
| N. tubacum (Tobacco) | TEV | crtE, crtB, crtI (VIGO) | Leaf | [44] |
| N. benthamiana | TEV | CCD2, CCD4 | Leaf | [99] |
| Ocimum basilicum (Sweet basil) | TRV | PDS | Leaf | [100] |
| Olea europaea (Olive) | TRV | PDS, ChlI | Leaf | [101] |
| Petunia | TRV | PDS | Leaf | [102] |
| Piper cubeba (Link) | TRV | PDS | Leaf | [103] |
| Pisum sativum (Pea) | PEBV | PDS | Leaf and stem | [82, 90, 105] |
| Prunus dulcis (Almond) | ALSV | PDS | Leaf | [60] |
| Prunus persica (Peach) | TRV, ALSV, PNRV | CCD4, PDS | Fruit | [50, 60] |
| Pyrus sp. (Pear and Japanese pear) | ALSV | PDS | Leaf | [56] |
| Rosa sp. Rose | ALSV, TRV | PDS | Leaf | [106, 107] |
| Senecio cruentus | TRV | PDS | Leaf and flower | [108] |
| Solanum lycopersicum L. (Tomato) | TRV, BCTV, DNAβ-, TYLCVV, ALSV | PDS, PDS, ZDS, ZISO, CRTISO, ChlI | Leaf and flower and fruit | [63, 67, 82, 109] |
| Solanum melongena (Eggplant) | TRV | PDS, ChlI | Leaf | [110] |
Table 1. Continued

| Plants | Viruses used for developing VIGR system | Gene Regulated (All are silenced unless indicated) | Plant tissue | Reference |
|--------|----------------------------------------|--------------------------------------------------|--------------|-----------|
| Solanum tuberosum (Potato) | PVX, TRV | PDS | Leaf and tubers | [111, 112] |
| Spinacia oleracea L. (Spinach) | BCTV, TRV | PDS | Leaf | [63, 113] |
| Thalictrum sp. | TRV | PDS | Leaf and flower | [114] |
| Vitis vinifera (Grape) | GVA, ALSV, GLRaV-2 | PDS | Leaf | [115–117] |
| Withania somnifera | TRV | PDS | Leaf | [118] |
| Zingiber officinal (Ginger) | BSMV | PDS | Leaf | [119] |
| Zucchini | ZYMV | CrtB (VIGO) | Fruit | [89] |

its function in controlling carotenoid biosynthesis. The decrease in carotenoid content might have been caused by minor off-target silencing of PSY1, however given that off-target homology between the PSY3 fragment and PSY1 are low, a role for PSY3 in mediating carotenoid biosynthesis in fruits cannot be excluded [109]. PSY homologs have been shown to have functional redundancy in different plant species. For example, when PSY1 was silenced in detached C. annuum fruit pericarp, silenced fruits were orange in colour and still synthesized a low level of carotenoids [75]. Fruits developed an albino phenotype and became devoid of carotenoids only when the PSY2 gene was silenced in the natural psy1 mutant pepper variety “Micropep yellow” [129]. Moreover, a colour complementation assay testing enzymatic activities of PSY in Escherichia coli revealed that PSY1 and PSY2 have the same enzymatic function and additionally, PSY1 is significantly overexpressed in the psy1 mutant plants. These findings emphasised the active redundancy between PSY homologs and highlighted the utility of VIGS in delineating gene functions and interactions between gene homologs among diverse horticultural crops.

Silencing of PSY reduces carotenoid content since this is the key rate-limiting bottle neck in carotenogenesis. PSY silencing causes an albino phenotype in fruits, making it an obvious visual reporter for the development and confirmation of VIGS expression systems [109]. Since plants can have multiple homologs of PSY with tissue specific expression, this adds another step to identify the correct gene homolog for targeted silencing [84]. Attempts to silence both PSY and PHYTOENE DESATURASE (PDS) in California poppy leaves and flowers using partial gene sequences, successful caused silencing of both genes in leaves, but only PDS was silenced in flowers. The inability of TRV-PSY to induce silencing in flowers could be accounted for by the presence of another PSY homolog specifically expressed in flowers. Therefore, the different activities exerted by different PSY homologues can complicate targeted silencing and limit its effectiveness as a visual reporter in assessing the successful engineering of a viral vectors in planta.

Phytoene undergoes a series of consecutive desaturation and isomerization reactions to extend the conjugated double bond in the C₄₀ backbone. This process is initiated by PDS, which desaturates 15-cis phytoene at 11–12 and 11′-12′ in two consecutive steps to produce 15,9′-di-cis phytofluene and 9,15,9′-tri-cis ζ-carotene, respectively [121]. Most plant species harbor only a single copy of PDS [130]. PDS is the most widely used reporter for VIGS as it provides a cheap and quick means of observing a visual marker change in tissue pigmentation (bleached, white or albino phenotype) and hence success, that accurately reflects the functionality of VIGS technology in planta (Table 1). VIGS of PDS expression rate-limits carotenoid biosynthesis to generate a biochemical loss of downstream carotenoids, like that of silencing PSY. Silencing PDS in leaves of tomato, cassava, pepper, petunia and Citrus sp. and in flowers of tomato and California poppy reduced PDS transcript levels, total carotenoid and chlorophyll content, leading to white sectors in leaves and flowers [35, 73, 84, 102, 131, 132]. Even if PDS silencing becomes less efficient during fruit cell division and expansion, white sectors that developed during the immature stages will later turn yellow during tomato fruit ripening and hence report VIGS [109, 132, 133]. The reduction in PDS transcript levels in TRV-VIGS-infected fruit can cause a severe reduction in desaturation of 15-cis phytoene into downstream carotenones causing a 2-fold increase in phytoene levels, and 95% and 50% reduction in lycopene and total carotenoid content, respectively [109].

ζ-CAROTENE DESATURASE (ZDS) desaturates 9,9′-di-cis-ζ-carotene at carbon positions 7 and 8 to produce 7,9,9′-tri-cis-neurosporene and at carbon positions 7′ and 8′ to produce 7,9,7′,9′-tetra-cis-lycopene (prolycopene) [134, 135]. Silencing of ZDS enhances the accumulation of 9,9′-di-cis-ζ-carotene and to a lesser extent, phytofluene and phytoene, causing a reduction in all-trans-lycopene (lycopene), the major carotenoid present in ripe tomato fruit [84, 109]. In contrast, ZDS silencing was also shown to cause a 30% increase in total carotenoid content in tomato fruits [84, 109]. In California poppy petals, silencing of ZDS reduced the total carotenoid content by 16-fold and triggered bleaching in flower petals [84]. Differences in the effect of ZDS silencing on the total carotenoid content in leaf, flower or fruits are likely due to the type and developmental stage of the plastid. There could also be differences in the biochemical activity of ZDS and/or feedback triggered by aberrant metabolite...
accumulation that alters carotenoid biosynthesis in different plant species and/or within plant tissues.

ZETA-CAROTENE ISOMERASE (ZISO) and CAROTENOID ISOMERASE (CRTISO) isomerise tri-cis-\(\gamma\)-carotene into di-cis-\(\gamma\)-carotene and tetra-cis-lycopeno (prolycopeno) into all-trans-lycopeno, respectively [134, 136, 137]. In the presence of a photosensitizer, light can mediate the photoisomerization of the cis-carotene bonds in the absence of ZISO or CRTISO activity [3]. Under light-limiting environmental conditions such as those conferred by a shorter photoperiod, CRTISO enzyme activity remains critical to ensure sufficient carotenoid biosynthesis and control the production of an apocarotenoid signal that regulates plastid development [138]. In tomato fruits, VIGS of ZISO and CRTISO did not significantly alter total carotenoid content, yet did alter carotenoid composition [109]. The differences in carotenoid composition between control and CRTISO silenced fruits were similar to that between wild type tomato (Rutgers) and the tangerine mutant (t3002) that has reduced CRTISO activity [139]. VIGS of ZISO and CRTISO resulted in the accumulation of its preceding desaturates’ substrate, indicating that PDS/ZISO and ZDS/CRTISO form functional catalytic units, where repression of either enzyme within each unit can lead to accumulation of the first substrate isomerised by either enzyme [109]. These findings reveal how utilising VIGS can provide valuable insights into the sequential biosynthesis of cis-carotenes and highlight the importance of ZISO and CRTISO in controlling carotenoid isomerisation in the absence, as well as presence of light.

The addition of either two \(\beta\)-ionone rings or \(\beta-\epsilon\)-ionone rings to all-trans-lycopeno through cyclization bifurcates the carotenoid pathway into alpha- and beta-branches. \(\beta\)-carotene and \(\beta-\beta\) xanthophylls, zeaxanthin, antheraxanthin, violaxanthin and neoxanthin, are generated by action of LYCOPENE \(\beta\)-CYCLASE (\(\beta\)-LYC), \(\beta\)-CAROTENE HYDROXYLASE (\(\beta\)-OHase), ZEAXANTHIN EPOXIDASE (ZEP) AND NEOXANTHIN SYNTHASE (NXS) (Fig. 1). \(\alpha\)-carotene and formation of \(\epsilon-\epsilon\)-xanthophylls; zeinoxanthin and lutein, are generated via a sequential activity by LYCOPENE EPSILON-CYCLASE (\(\epsilon\)-LCY), \(\beta\)-LYC and \(\beta\)-OHase (Fig. 1). The cyclic carotenoids provide a significant carbon sink of metabolites that are stored in leaves, fruits and flowers of different fruit and ornamental crops. VIGS systems have not extensively been used to study the function of these downstream carotenoid biosynthetic enzymes that synthesise xanthophylls and \(\alpha/\beta\)-carotene metabolites. Silencing of \(\epsilon\)-LCY in tobacco (Nicotiana tabacum) reduced downstream carotenoids and redirected carotenoid flux towards the \(\beta\)-branch in the pathway [98]. Cyclic carotenoids can be converted into specific carotenoids in different plant species such as capsanthin/capsorubin in pepper fruits and the lily flower (Lilium leichtini), astaxanthin in the flowers of Adonis aestivalis, and eschscholtzaxanthin in flowers from the California poppy [140, 141]. Even though the exact mechanism of biosynthesis of these carotenoids has not been completely elucidated, silencing of upstream carotenoid biosynthesis genes such as PSY, \(\beta\)-LYC or \(\beta\)-OHase in pepper fruit, or PDS, ZDS, \(\beta\)-OHase or ZEP in California poppy, was able to reduce the total content of capsanthin/capsorubin and eschscholtzaxanthin, respectively [75, 84].

VIGR of carotenoid degradation

Carotenoids are degraded enzymatically and non-enzymatically to produce various apocarotenoids that have important biological functions [142–144]. The enzymatic degradation of carotenoids occurs through oxidative cleavage of double bonds by CAROTENOID CLEAVAGE DIOXYGENASE (CCD) enzymes [145]. The CCD gene family in Arabidopsis contains four CCD homologues (CCD1, 4, 7, 8) and five NCED homologues (NCED2, 3, 5 and 9) [146]. Some CCD genes have undergone gene duplication events in different plants to produce similar variants such as CCD1A and CCD1B in tomatoes [147] and CCD4A, CCD4B and CCD4C in Crocus sativus [148, 149]. CCDs degrade carotenoid substrates to produce apocarotenoids [146]. VIGR has been used to demonstrate that carotenoid degradation and apocarotenoid biosynthesis pathways could be exploited to biofortify fruits and produce valuable secondary metabolites. In a white peach variety “Akatsuki” the total content of carotenoid was increased approximately 2.5-fold when the CCD4 gene was transiently silenced using the TRV-based VIGS system [50]. The silenced part of the fruit mesocarp displayed a yellow pigmentation because of increased levels of violaxanthin, lutein, zeaxanthin, \(\beta\)-cryptoxanthin and \(\beta\)-carotene. The transient heterologous overexpression of C. sativus CCD2 and Buddleja davidii CCD4 in N. benthamiana using a tobacco etch potyvirus-based VIGO system led to the synthesis of highly valued carotenoid-derived spices, such as crocins and picrocrocin by 0.2% and 0.8% of leaf dry weight, respectively [99]. Apocarotenoid levels were further increased to 0.35% of leaf dry weight when \(\beta\) ananatis PSY and saffron \(\beta\)-OHase were overexpressed together with the aforementioned genes. Collectively, these studies demonstrate how VIGR has served as an effective tool to decipher strategies (biotechnology or breeding) towards biofortifying commercially important carotenoids and apocarotenoids for industrial application.

VIGR of carotenoid storage and sequestration

Fruits and leaves are composed of chromoplast and chloroplast plastid types that provide suitable sinks to store carotenoids [6]. The differentiation of a proplastid into these plastid types is controlled by genetic factors, environmental conditions, and the cellular level of carotenoids. VIGO of the bacterial carotenoid biosynthesis gene CritB using Lettuce mosaic virus and Zucchini yellow mosaic virus vectors in lettuce and zucchini, respectively, increased carotenoids and caused differentiation of chloroplasts into chromoplasts in green tissues [89]. The
accumulation of higher amounts of phytoene caused the chloroplasts to lose their photosynthetic functionality and, upon conversion of phytoene into downstream carotenoids, a retrograde apocarotenoid signal was proposed to trigger the differentiation of the chloroplasts into chromoplasts thereby providing a better sink to store carotenoids.

Plastid number (biosynthesis and differentiation) and size are genetically regulated by a network of genes and transcription factors (TFs). Transient silencing of capsicum *ARABIDOPSIS PSEUDO RESPONSE REGULATOR 2-LIKE* (PRR2) with TRV vectors in capsicum fruit pericarp caused the development of pale-yellow fruit compared to the yellow control fruit [150]. Transmission electron microscopy of capsicum genotype with non-functional PPR2 mutation indicated that PPR2 positively regulates plastid number and overall size by increasing plastid compartment size, maintaining thylakoid and granum structure in chloroplast, and enlarging plastoglobules in the chromoplast [150]. These results are in accordance with where tomato PPR2 was transgenically overexpressed [151]. The *DIFFERENTIATION 1* (DET1) – CULLIN 4 (CUL4) multiprotein complex for proteosome degradation [154, 155]. The loss-of-function of DDB1 and DET1 in *high pigment 1* (hp1) and *high pigment 2* (hp2) tomato varieties, respectively, leads to a higher plastid number and size resulting in enhanced chlorophyll and carotenoid levels in immature and ripe tomato fruits [156, 157]. VIGS of DDB1, DET1 or CUL4 in *N. benthamiana* increased accumulation of GLK2, revealing that plastid number and pigment content in hp1 and hp2 could be due to increased activity of GLK2 [158]. These findings established by using VIGS have deepened our understanding of linkages between carotenoid sequestration and plastid biogenesis.

### Regulation of fruit ripening and carotenoid accumulation

Carotenoid biosynthesis and storage in ripening climacteric fruits is intricately regulated by ethylene biosynthesis and signalling mechanisms [159]. In tomato, VIGS of key genes regulating the ethylene biosynthesis and signalling 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2 (ACS2), ACS4 and 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE 1 (ACO1) reduced carotenoid synthesis leading to the development of green patches in silenced fruit that reflected an inhibition of ripening processes [160]. PROTEIN RIPENING INHIBITOR (RIN), SQUAMOSA PROMOTER BINDING PROTEIN COLOURLESS NON-RIPENING (SPL-CNR), HOMEODOMAIN-LEUCINE ZIPPER 1 (HB-1) and NACs (NO APICAL MERISTEM, ATAF, CUP-SHAPED COTYLEDON FAMILY PROTEIN) are TFs that regulate ethylene biosynthesis and affect carotenoid metabolism [161]. RIN binds to the promoters of ACS2 and ACS4 to promote ethylene biosynthesis [160, 162]. Silencing of RIN in tomato using TRV-based vectors reduced transcript levels of ACS2 and ACS4 (~70%) and ACO1 (~40%) [160]. Silencing SPL-CNR with Potato virus x-VIGS and NORK-like1 with the TRV-VIGS system in tomato inhibited ripening causing patches of pale green unripe sectors to develop [163, 164]. VIGS coupled with an in vitro retardation assay of HB-1 in tomato indicated that HB-1 binds to the promoter of ACO1 to regulate conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) into ethylene [165]. VIGS of NAC4 and NAC9 TFs in tomato fruit suppressed ethylene biosynthesis by reducing expression of ACS2, ACS4, ETHYLENE RECEPTOR 4 (ETR4) and NEVER RIPENING (NR, involved in ethylene perception), and correspondingly decreased total carotenoid content due to a reduction in expression of PSY1, PSY2, PDS, ZDS, ZISO and CRTISO [166]. VIGS of tomato AGAMOUS-LIKE1 (TAGL1) reduced carotenoid levels in ripening fruit and displayed a thinner pericarp as has been seen in tomato fruits where TAGL1 was silenced using a transgenic approach [167]. Transcriptomic analysis of transgene-silenced TAGL1 as well as a transient promoter binding assay indicated that TAGL1 promoted carotenoid accumulation by activating expression of ACS2 [167]. Similarly, VIGS of ethylene responsive-WRKY 16, 17, 53 and 54 in tomato fruits reduced ripening of fruits that developed greenish-yellow fruit indicating lower carotenoid levels. These phenotypes resemble those caused by 1-methylcyclopropene (1-MC, inhibitor of ethylene action) treatment of fruits. RNA-seq analysis of ethylene and 1-MC treated fruit, along with yeast-2-hybrid assay, indicated that WRKY TFs promote carotenoid accumulation by upregulating the expression of PSY1 and PDS [168]. These aforementioned VIGR studies further evidence an integrated regulation between ethylene and carotenoid biosynthesis during climacteric fruit ripening.

Carotenoid metabolism during ripening of non-climacteric fruits can be controlled by abscisic acid (ABA)-mediated regulation [169]. ABA is an apocarotenoid formed by the catalytic sequential degradation of violaxanthin and neoxanthin by 9-CIS-EPOXYCAROTENOID DIOXYGENASE (NCEDs), SHORT-CHAIN DEHYDROGENASE/REDUCTASE (SDR/ABA2) and ABSCISIC ACID HYDROGENASE (AAO). ABA plays an important feedback regulatory role in controlling carotenogenesis. For example, silencing of NCED1, the PYRABACTIN RESISTANCE 1 (PYR1) ABA receptor, and other signalling components such as the MAGNESIUM CHLORIDE CHLORIDE (CHLH/ABA) and ABA-SENSITIVE 4 (ABI4) using TRV-based VIGS caused a reduction in carotenoid accumulation and delayed/inhibited strawberry fruit ripening [49, 170–172]. These studies also found that sucrose positively regulated ABA accumulation and fruit ripening. That is,
exogenous application of sugar increased endogenous ABA and accelerated ripening in strawberry, whereas silencing of the sucrose transporter SUT1 via RNAi decreased the level of sucrose, ABA, fruit ripening and total carotenoid levels [49, 170].

The roles of ethylene and ABA in regulating ripening processes in climacteric and non-climacteric fruits appear to be interrelated in controlling ripening and carotenoid metabolism [166, 173, 174]. In hot pepper (Capsicum frutescens, a non-climacteric fruit), VIGS of ABA biosynthesis genes NCED1 and NCED3 and the ethylene biosynthesis gene AC03 had the opposite effects on fruit carotenoid content [175]. That is silencing of NCED1 and NCED3 increased total carotenoid by upregulating expression of PSY, PDS, ZEP, ZDS, and CAPSANTHIN-CAPSORUBIN SYNTHASE (CCS), whereas silencing of AC03 reduced total carotenoid by downregulating the aforementioned carotenoid biosynthesis genes [175]. In strawberry fruits, VIGS of the ethylene biosynthesis gene S-ADENOSYL-L-METHIONINE SYNTHETASE (SAMS) and ethylene signalling component CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) inhibited ripening and development of red pigments [176]. In tomato, silencing of the ABA biosynthesis gene NCED1 suppressed lycopene production by silencing PSY1 and PDS, inhibited ripening-related fruit softness by downregulating PG1 and EXP1, and yet increased ethylene biosynthesis and corresponding gene expressions (e.g. ACS2, ACS1, ETR3, ETHYLENE RESPONSE FACTOR 2; ERF2) [177]. In contrast, silencing of the CYTOCHROME P450 707A family gene CYP707A2 (involved in ABA degradation) resulted in early ripening of tomato fruits. The transcript levels of ACS2, ACS1, ETR3, EXP1, and PSY1 reached the highest level in silenced fruits three days earlier than control fruits. These results clearly indicate that there is a crosstalk between ethylene- and ABA-mediated ripening mechanisms [175, 177]. There are gaps in understanding how ethylene- and ABA-mediated ripening mechanisms are interconnected with carotenogenesis. The fast and easy application of VIGR expression systems in different fruits has advanced our understanding of ripening-mediated carotenoid metabolism. Developing VIGR systems for other crops where genetic manipulation strategies are not always efficient, such as mangoes, citrus and avocado, will further improve our understanding of carotenogenesis in these horticultural tree crops.

**Manipulating substrate supply alters carotenogenesis process**

Carotenoid biosynthesis is reliant upon the upstream methyerythritol phosphate (MEP) pathway for the supply of isoprenoid substrates, and disruption of the MEP pathway can block carotenogenesis [178]. For example, disrupting the MEP pathway by VIGS of 4-(CYTIDINE 5'-DIPHOSPHO)-2-C-METHYL-D-ERYTHRITOL KINASE (CMK), 4-HYDROXY-3-METHYL-2-ENYL-DIPHOSPHATE SYNTHASE (HDS) and 4-HYDROXY-3-METHYL-2-ENYL-DIPHOSPHATE REDUCTASE (HDR) in N. benthamiana caused an approximate 72% decrease in total carotenoid levels [179]. Silencing expression of HDS and HDR using TRV led to the development of albino leaves in N. benthamiana and decreased (~96%) chlorophyll and carotenoid pigment levels [180]. These results were consistent with findings from other studies where mutations or T-DNA knockout of MEP pathway genes, 1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE (DXS), 1-DEOXY-D-XYLULOSE-5-PHOSPHATE REDUCTOISOMERASE (DXR), 4-DIPHOSPHOCYTIDYL-2-C-METHYLERITHRITOL SYNTHASE (CMS), CMK, 2-C-METHYL-D-ERYTHRITOL-2,4-CYCLODIPHOSPHATE SYNTHASE (MCS) and HDS in Arabidopsis lead to development of an albino phenotype as a result of decreased chlorophyll and carotenoid content [181–185]. Therefore, VIGR studies can be utilised effectively to manipulate the expression of MEP pathway genes and hence enzyme functions that catalyse the supply isoprenoid substrates for carotenogenesis.

The carotenoid biosynthesis pathway is interlinked with numerous other pathways that require the plastid derived GGPP as a common precursor for biosynthesis. The supply of common precursors is paramount to maintain a natural flux of GGPP to maintain cellular homeostasis, and manipulation of any one pathway can create a cascade of changes to other metabolic pathways. For example, increasing GGPP flux into the carotenoid pathway by overexpressing PSY in N. benthamiana and tomato using transgenic approaches caused an increase in total carotenoid content [186, 187]. However, this reduced the availability of GGPP for gibberellic acid (GA) biosynthesis pathway, which in turn reduced GA biosynthesis and caused dwarfism. Similarly, increasing the pool of GGPP for other pathways by limiting its flux into carotenogenesis using TRV-PSY in N. benthamiana increased paclitaxel biosynthesis and caused a 2-fold accumulation of taxadiene, the first committed product of the paclitaxel pathway [188]. Therefore, manipulating the upper MEP pathway and GGPP biosynthesis using VIGR adds further value to our knowledge of how upper rate-limiting steps effect the supply of substrates for carotenoid biosynthesis.

**Scope, advantages and limitations of VIGR for horticulture advancement**

The VIGR system has several advantages over other biotechnological techniques such as chemical mutagenesis, transgene insertion, RNA interference or CRISPR/Cas9 gene editing, regarding functional genomic approaches involving the silencing or overexpression of target genes. For example, the transient VIGR system can be used to manipulate the expression of any gene, including those that become lethal to the plant host when transgenically altered. The VIGR system can be used to specifically silence genes in detached fruits from horticultural crops, thereby reducing the
need to grow large trees or climbing plants within an approved arrangement biosecurity containment facility designated for VIGR research. VIGR does not require transgene transformation or tissue culture to alter transcript levels of target genes and hence, VIGR can be performed rapidly and in a cost-efficient manner. These advantages of VIGR makes it a tool of choice for conducting functional genomic studies in non-model horticultural crops (Fig. 3).

VIGR provides a reliable high throughput platform to advance both functional genomic studies in non-model horticultural crops producing fruits, vegetables, nuts, or ornamental features. Functional genomics in non-model crops has been expanded through recent advances in de novo assembly of plant genomes, such as pineapple [189], almond [190, 191], mango [192], macadamia [193] and avocado [194] where new genomic knowledge can now lead to improved functional annotation. VIGR in combination with phylogenetics, metabolomics, transcriptomics and/or genomic approaches can advance gene characterisation and protein function in non-model species (Fig. 3A). Such a technique was used in citrus to identify OXIDOSQUALENE CYCLASE and CHALCONE SYNTHASE genes that regulate limonoid and flavonoid synthesis, respectively [195, 196]. As another forward genomics approach, VIGR was used to refine the C1 locus (PSEUDO RESPONSE REGULATOR 2; PRR2) responsible for fruit colour in pepper and the R1 locus (GhPSY) responsible for red pigment plant phenotype in cotton (Fig. 3A) [150, 197]. VIGR of differential genes obtained from a suppression subtractive hybridization cDNA library identified the SINA P7 gene as the candidate associated with reduced lycopene pigment formation in tomato fruit. When SINA P7 was silenced using VIGS there was a downregulation in POR and PORA expression leading to an impairment in chloroplast development and reduced lycopene accumulation in tomato fruits [198]. These aforementioned examples, highlight how VIGR can be used to fast forward genetic cDNA library screening and hence functional genomics [199].

VIGR technologies have advanced considerably over the past two decades. Major drawbacks associated with VIGR technology have been limited to a small number of effective viral vectors, narrow host range specificity and off-target silencing. This has been resolved mostly through the development of new VIGR vectors capable of infecting a wider host range, facilitating homologous recombination multiple cloning sites, and improved agrobacterium/viral vector inoculation methodologies [200]. Different web-based programs such as Sol Genomics Network (SGN) VIGS and pssRNAIt have been developed to simplify the process of identifying gene sequence fragments that are unique and avoid off-target silencing incidents [201, 202]. However, there are still further limitations of VIGR. For example, the spread of systemic RNA-mediated signals and suppression of viral infection are not ubiquitous among all plants. Experiments silencing PDS in fruits and leaves of tomatoes and Capsicum sp. have shown that VIGS does not silence genes homogeneously in all plant tissues. In California poppy, the spread of PDS silencing was faster when the shoot apical meristem and surrounding leaves were inoculated in comparison to when lower leaves were inoculated [35]. The uneven silencing can make it difficult to collect VIGR tissues for metabolic and gene expression studies, especially if there is no obvious pigmentation phenotype to score. Several species-specific reporter systems such as Delila and Rosea1 (Del/Ros1)-based reporter system for tomatoes [203], anthocyanin accumulating purple reporter system for tobacco [204], and the generic green fluorescent protein (GFP)-based reporter system [205] can help to overcome phenotyping challenges and further refine VIGR technologies.

The use of VIGO to transiently overexpress target genes regulating carotenoid metabolism or other biological processes is rather sparse compared to the application of VIGS (Fig. 1; Table 1). The biggest obstacle towards developing VIGO systems has been the limitations surrounding the insert size that can be delivered and retained by the viral vector. Different techniques have been implemented to increase the cargo capacity for foreign gene insertion into viral vectors that allow larger or multiple proteins to be expressed in planta. In a Barley stripe mosaic virus based VIGR vector, increasing the partite system from a three-partite to four-partite vector enhanced the capacity of the viral vector to overexpress genes up to 2.1 kb [206]. Eliminating viral coat protein or movement protein coding sequences (deconstructed virus vector strategy) in viral vectors can also increase the cargo capacity of vectors [207, 208]. For example, deletion of the viral Nib cistron from TEV increased the cargo capacity or space to harbor foreign sequences and enabled the simultaneously overexpress of three bacterial carotenoid biosynthesis genes (crtE, crtB and crtI) in transgenic N. benthamiana haboring the Nib cistron [43]. Overexpression of these three bacterial carotenoid proteins increased endogenous carotenoid levels in the cytosol and reduced chlorophylls to result in a bright yellow pigmentation in N. benthamiana leaves, providing a useful colour-based reporter system to track the infection dynamics of plant viruses [44]. This exemplifies the utility of viral vectors for multiple gene expressions that drive complex foreign biochemical pathways.

The latest development of Potato virus x, Foxtail mosaic virus, Barley yellow striate mosaic virus and Sonchus yellow net rhado virus-based viral vectors are capable of overexpressing large gene sequences, including an ∼4.5 kb CRISPR-Cas9 cassette in N. benthamiana, which is an important break-through in the field allowing researchers to conduct virus-induced genome editing (VIGE) [46, 209–211]. Furthermore, DNA-free genome editing of Nicotiana benthamiana has been achieved using TEV and Potato virus X (PVX) vectors to simultaneous overexpress Cas12a nuclease and guide RNAs to mediate VGE without the need of transgenic processing elements.
Application of virus induced gene regulation

(a) Functional genomics
- One or multiple genes cloned into the viral vector
- cDNA libraries of genes cloned in viral vectors
- Viral vectors transformed into Agrobacterium
- Inoculation of viral vectors into any plant tissue
- Silencing/over-expression of target gene in plants
- Phenotypic and molecular characterisation
- Reverse Genetics
- Forward Genetics

(b) Crop improvement
- Clone full FT gene (to overexpress) and partial TFL gene (to silence) into viral vector
- Inoculation of viral vector into plant tissue
- Precocious flowering induced in plantlets
- Selfing/crossing
- Screen germplasm for important traits
- Collection of next-generation seeds
- Accelerate traditional breeding

(c) New genetic cultivars
- CRISPR-Cas9 cassette and guide RNA sequence cloned into viral vector
- Inoculation of viral vector into plant tissues allows CRISPR-Cas9 to guide editing of genome
- Select for transformed cell with edited genome
- Eliminate virus from new plant growth
- Tissue culture
- Seed collection
- Vegetative propagation
- Mutant plant for functional genomics
- Non-transgenic plant, new genetic variety

Figure 3. Application of virus-induced gene regulation (VIGR) in advancing functional genomics and crop improvement in horticultural crops. (a) Functional genomics. VIGS and VIGO can be utilised in forward and reverse genetic screening approaches. Partial fragments or full-length coding sequences of the target gene (single, multiple or cDNA library) are cloned into the viral genome within a binary vector and transiently expressed in plants. Phenotypic and molecular assays are performed to characterise gene functions in infected tissues. (b) Crop improvement. Simultaneous overexpression of flowering locus T (FT) and silencing of terminal flower locus (TFL) using the VIGR system can trigger precocious (early) flowering. Flowers that are self- or cross-pollinated will develop fruits and seeds. Phenotyping and molecular analysis of flowers, fruits and seeds can be undertaken in combination with traditional breeding strategies to speed up the identification of germplasm harboring desirable genetic traits (e.g. pathogen resistance, self-compatibility). Virus-free seeds can be isolated to advance the next breeding cycle. (c) New genetic cultivars. VIG gene-editing systems have been deployed to identify non-transgenic mutant plants for functional genomics and engineer elite genetic cultivars. Overexpression of the CRISPR-Cas9 and RNA guide system can edit a heritable mutation in the genome that will be inherited during mitosis and cell division. The virus can be eliminated from the plant through heat/cold treatment. Selection and confirmation of the transformed cell or genetic mutation in virus-free plants can be obtained from seeds, tissue culture cell lines, or by vegetative propagation. Systemic spread of siRNA for silencing. Systemic spread of target gene with viral component for overexpression.
thereby allowing transgenerational inheritance of mutations to seeds from infected plants without inclusion of viral components [212, 213]. VIGE systems expand our toolbox to create heritable mutations without requiring the development of a transgenic organism (Fig 3C). This has several advantages over transgenic or CRISPR/Cas9-mediated gene overexpression approaches, as VIGE does not require genotyping and self- or back-crossing. Such non-transgenic plants could be used for functional genomics studies and/or released as new crop varieties that are more nutritious and productive. For example, editing of the genes/promoters of CRTISO, ε-LYC and β-LYC in mango through VIGE could fast-forward development of a non-transgenic pink mango fruit altered in colour and enriched in lycopene. Similarly, VIGE of PSY and β-LYC could lead to a new generation of golden coloured rice enriched in provitamin A (β-carotene). Further research is necessary to determine if the large Cas9 could be replaced with smaller Cas proteins such as Cas9 [214], as this could enable the remaining cargo capacity of the VIGE vector to be used to edit multiple genes when attempting to modify complex traits. The capability of VIGE to cause heritable non-transgenic mutations and induce gene overexpression will provide unprecedented opportunities to transform future functional genomics studies and plant breeding endeavours in horticultural crops.

Carotenoids provide colour to fruits and flowers of horticultural trees and add economic value by providing essential micronutrients such as provitamin A. However, carotenoid metabolism among other important traits such as shoot branching and precocity have scarcely been studied in horticultural fruit tree crops. Long juvenile phases can range between 3 to over 20 years thereby increasing the amount of time and resources required to conduct functional genomic studies in tree crop species [215]. Transient overexpression of positive regulators of flowering such as FLOWERING LOCUS T and/or the downregulation of negative regulators of flowering such as TERMINAL FLOWERING LOCUS 1 in apple, pear and citrus have demonstrated how VIGR can be utilised to induce precocious flowering in juvenile horticultural tree plants and reduce the juvenile phase from several years to less than a few months [56, 59]. Pollination following virus-induced flowering can result in the development of fruit with fertile, non-transgenic seeds like that of non-VIGR infected plants. Successful induction of precocious flowering has opened new opportunities to fast-forward functional genomic studies to study carotenogenesis and associated phytohormone pathways such as shoot branching architecture (e.g. strigolactones) or drought responses (e.g. abscisic acid). VIGR can speed up germplasm development and plant breeding programs through inducing early flowering/fruiting and enable the rapid identification of allelic gene functions, new trait loci or novel gene functions controlling these commercially important traits in fruit trees (Fig. 3B). The advantages of virus-induced flowering: high-throughput germplasm screening, rapid functional genomics studies and reduced breeding cycle, can overcome current challenges and provide new avenues to advance the genomics of horticultural tree crop breeding.

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CIC and LP conceived review and visualised perspectives. LP wrote the article and prepared figures with assistance from primary supervisor CIC. Co-supervision of LP was provided by JP and AP. All authors reviewed, edited, provided critical input and approved the manuscript.

Conflicts of interest statement
We declare there are not conflicts of interest.

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We declare there are not conflicts of interest.

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