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Virus-induced gene silencing for Asteraceae—a reverse genetics approach for functional genomics in *Gerbera hybrida*

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Summary

Virus-induced gene silencing (VIGS) is a natural defence mechanism in plants which leads to sequence-specific degradation of viral RNA. For identifying gene functions, *Tobacco rattle virus* (TRV)-based VIGS has been applied for silencing of endogenous genes in many plant species. *Gerbera hybrida* (Asteraceae) has emerged as a novel model for studies in flower development and secondary metabolism. For this highly heterozygous species, functional studies have been conducted through reverse genetic methods by producing stable transgenic lines, which, however, is labour-intensive and time-consuming. For the development of TRV-based VIGS system for gerbera, and for the first time for an Asteraceaeous species, we screened several gerbera cultivars and optimized the agroinfiltration methods for efficient silencing. Gene fragments for gerbera phytoene desaturase (GPDS) and Mg-chelatase subunits (GChl-H and GChl-I), expressed from a TRV vector, induced silencing phenotypes in leaves, scapes, and involucral bracts indicating their feasibility as markers for green tissues. In addition, robust silencing symptoms were achieved in gerbera floral tissues by silencing the anthocyanin pathway gene for chalcone synthase (GCHS1) and a gerbera B-type MADS-box gene *globosa* (*GGLO1*), confirming the phenotypes previously observed in stable transgenic lines. Unexpectedly, photobleaching induced by *GPDS* and *GChl-H* or *GChl-I* silencing, or by the herbicide norflurazon, resulted in silencing of the polyketide synthase gene *G2PS1*, which has no apparent connections to carotenoid or chlorophyll biosynthesis. We have shown feasibility of VIGS for functional studies in gerbera, but our results also show that selection of the marker gene for silencing must be critically evaluated.

Introduction

Plants have evolved to counter virus infection by recognizing and processing the double-stranded RNA intermediates of virus replication (Meister and Tuschl, 2004; Purkayastha and Dasgupta, 2009). This leads to sequence-specific RNA degradation, known as RNA silencing (Baulcombe, 2004). When a host gene fragment is incorporated into an engineered virus, degradation of the host-derived sequence, as part of the viral RNA, targets also the corresponding host mRNA to post-transcriptional silencing. This process has been designated as virus-induced gene silencing (VIGS) (van Kammen, 1997). Because VIGS allows targeted down-regulation of particular host genes, the potential of VIGS as a functional genomics tool was quickly recognized (Baulcombe, 1999). VIGS has been widely used for identifying gene functions in species of many taxa, including those of Solanaceae (Brigneti *et al.*, 2004; Chen *et al.*, 2004; Chung *et al.*, 2004; Kumagai *et al.*, 1995; Liu *et al.*, 2002), *Arabidopsis thaliana* (Brassicaceae) (Burch-Smith *et al.*, 2006; Wang *et al.*, 2006) and *Oryza sativa* (Poaceae) (Ding *et al.*, 2006; Purkayastha *et al.*, 2010). Because VIGS does not require the generation of stably transformed lines (Burch-Smith *et al.*, 2004), it is an especially appealing method for species that are lacking efficient tissue culture methods but nevertheless possess emerging need for functional studies, for example, along large-scale sequencing efforts or for comparative purposes. Intriguingly, the use of VIGS has expanded to many potential emerging model plants, such as *Papaver somniferum* and *Eschscholzia californica* (Papaveraceae) (Hileman *et al.*, 2005; Wege *et al.*, 2007), *Phalaenopsis amabilis* (Orchidaceae) (Lu *et al.*, 2007), *Aquilegia vulgaris* and *Thalictrum* sp. (Ranunculaceae) (Di Stilio *et al.*, 2010; Gould and Kramer, 2007; Kramer *et al.*, 2007), *Spinacea oleracea* (Amaranthaceae) (Golenberg *et al.*, 2009), *Jatropha curcas* (Euphorbiaceae) (Ye *et al.*, 2009), *Zingiber officinale* (Zingiberaceae) (Renner *et al.*, 2009), *Malus domestica*, and *Pyrus pyrifolia* (Rosaceae) (Sasaki *et al.*, 2011).

Gerbera hybrida (gerbera) is one of the most important ornamental species, with the total sales ranked 4th in cut flowers after rose, chrysanthemum, and tulip in Dutch auctions in 2011 (Plasmeijer and Yanai, 2012). The commercial varieties of gerbera are hybrids between two species, *G. jamesonii* and *G. viridifolia* (Hansen, 1999). They belong to the sunflower family Asteraceae that is one of the largest families of flowering plants. Besides gerbera, it includes also many other economically important genera such as *Helianthus* (sunflower), *Lactuca* (lettuce), *Cichorium* (endive, chicory), *Calendula* (marigold), and *Dendranthema* (chrysanthemum) (Judd *et al.*, 2002). Typical of Asteraceae, gerbera has a complex inflorescence, the capitulum that bears morphologically different types of flowers. This is a

trait that distinguishes Asteraceae from the conventionally used model species for flower developmental studies, such as *Arabidopsis thaliana* or *Petunia hybrida* that bear only single types of flowers in their inflorescences (Teeri *et al.*, 2006a). Already two decades of flower developmental studies have turned gerbera into a true Asteraceae model (Broholm *et al.*, 2008; Elomaa *et al.*, 1993, 1998; Helariutta *et al.*, 1996; Kotilainen *et al.*, 2000; Laitinen *et al.*, 2005; Tähtiharju *et al.*, 2012; Uimari *et al.*, 2004; Yu *et al.*, 1999). Furthermore, gerbera tissues are rich in many unique secondary metabolites, such as gerberin and parasorboside, which are important in protecting the plant against microbial attack and insect herbivores (Eckermann *et al.*, 1998; Koskela *et al.*, 2011; Ulla Anttila *et al.*, unpublished results). The highthroughput genomic techniques (Laitinen *et al.*, 2005) and ongoing large-scale sequencing efforts are constantly increasing the available collections of gerbera EST sequences (Teemu Teeri and Paula Elomaa, unpublished) and require efficient approaches to identify the functions of the corresponding genes. However, gerbera, like many other emerging models, lacks the ease of functional analysis typical to primary plant models. It is an outcrossing and highly heterozygous species and suffers from strong inbreeding depression, which makes forward genetic approaches for gene discovery impractical. For functional studies of gerbera genes, methods to produce stable transgenic lines through *Agrobacterium tumefaciens*-mediated gene transfer or by particle bombardment are established but are labour-intensive and time-consuming (Elomaa and Teeri, 2001; Elomaa *et al.*, 1993; Teeri *et al.*, 2006a,b). VIGS is an attractive alternative as it allows for rapid preliminary identification of gene functions without stable plant transformation (Burch-Smith *et al.*, 2004).

Tobacco rattle virus (TRV) is a bi-partite, positive-sense, single-stranded RNA virus with one of the broadest host ranges among plant viruses (Harrison and Robinson, 1978; MacFarlane, 1999). VIGS vectors developed from TRV (Liu *et al.*, 2002; Ratcliff *et al.*, 2001) have been used for gene function characterization in many plant species (reviewed by MacFarlane, 2010). TRV infection causes relatively mild disease symptoms and often induces intensive and uniform silencing phenotypes (MacFarlane, 2010; Ratcliff *et al.*, 2001). Importantly, not like most other RNA viruses, TRV can also infect meristematic tissues, which allows silencing of genes in the growth points (Ratcliff *et al.*, 2001). Infection of *Gerbera jamesonii* by TRV has been reported previously (Stouffer, 1965). The aim of this study was to develop a TRV-based gene-silencing method for the cultivated *Gerbera hybrida*. Through screening of gerbera cultivars for susceptibility to TRV, and by optimizing agroinfiltration methods, efficient gene knock-down phenotypes were achieved both in gerbera leaves and inflorescences. This is, to date, the first report of application VIGS in species of Asteraceae.

Results

Screening of gerbera cultivars for efficient VIGS response

Our initial VIGS experiments were carried out using the variety Terraregina, a gerbera cultivar that is routinely used in our laboratory for large-scale sequencing and stable genetic transformation. The *gerbera phytoene desaturase* (*GPDS*) gene was adopted as a VIGS reporter. For the construction of the *GPDS* VIGS vector, a 437-bp fragment of *GPDS* was amplified from cultivar Terraregina and cloned into TRV RNA2 VIGS vector (Liu *et al.*, 2002) to form TRV2:GPDS. The binary vectors for the expression of TRV RNA1 and TRV2:GPDS (Liu *et al.*, 2002) were transformed to *Agrobacterium tumefaciens* and introduced to the leaves of small gerbera plants using the traditional syringe-infiltration method (Kapila *et al.*, 1997) 5 weeks after potting. However, neither *PDS*-silencing phenotypes nor virus symptoms were observed in variety Terraregina.

To find TRV VIGS–sensitive cultivars, a screening experiment was carried out using 21 different gerbera cultivars (Table S1). Gerbera plantlets, multiplied *in vitro*, were syringe infiltrated on two fully expanded leaves 5 weeks after potting in soil (stage 2, Figure 1a). The tested gerbera cultivars showed large variation in TRV VIGS responses. By 30 days postinfiltration (dpi), gerbera plants from five cultivars (Grizzly, White Grizzly, President, Lamborghini, and Tucan) showed photobleached *PDS*-silencing symptoms in newly developed leaves (Figure 1b). The photobleached area per leaf varied from more than 50% of the whole leaf area to tiny patches (Table 1). In addition, the number of photobleached plants per cultivar varied from none to 12 of 18 treated plants (Table 1). Cultivar Grizzly exhibited the most intensive *PDS*-silencing phenotype, and the silencing symptoms in this variety appeared as early as 12 dpi. On the other hand, President was the most TRV-sensitive cultivar, and 12 of the 18 treated plants showed a photobleaching phenotype (Table 1). However, when infected with TRV, President plants displayed severe disease symptoms including epinasty and formation dark-green patterns in systemically infected leaves (Figure S1), which made this cultivar not optimal for VIGS. Plants from cultivar Terraregina, again, showed no silencing symptoms (Figure 1b). Both RNA1 and RNA2 of TRV were detected with RT-PCR in tissues of upper non-inoculated leaves from plants that showed photobleaching phenotypes (Table 1, Figure S2a), as expected. However, TRV RNAs were detected only in about one-third of the silenced plants from samples taken from the non-photobleached topmost leaves (Table 1, Figure S2b).

Optimizing conditions for TRV VIGS in gerbera

Next, we optimized several parameters that are likely to improve the efficiency of VIGS in gerbera. We first increased the density of the agrobacterium inoculum from OD_{600} 1 to OD_{600} 4. However, no difference on silencing efficiency was observed (data not shown). Next, because the developmental stage of the plant at the time of inoculation is critical for VIGS (Burch-Smith *et al.*, 2006; Hileman *et al.*, 2005), the optimal stage for VIGS of gerbera was studied. The gerbera plantlets were derived from tissue culture, and the early stages of vegetative development were divided into three age categories based on the number of days postpotting and the morphology of leaves. Plants at stage 1 were at maximum 4 weeks old. At this stage, all leaves of the plant were round in shape (Figure 1a). At stage 2, plants were about 5 weeks old, and the emergence of lobed-shaped leaves started (Figure 1a). At stage 3, plants started to grow very fast, and compared to earlier stages, all newly developed leaves were large, lobed in shape, and with long petioles (Figure 1a). Gerbera plantlets at stage 1 showed the highest sensitivity to TRV VIGS. For cultivar Grizzly, 11 of 12 syringe infiltrated plants showed intense PDS-silencing symptoms. Also, two of 12 infiltrated plants of cultivar Terraregina, which showed no silencing in the cultivar screen experiments (inoculated at stage 2), showed tiny photobleaching patches when inoculated at stage 1. When plantlets were syringe infiltrated at stage 2, much lower silencing efficiency was observed; five of 12 infiltrated Grizzly

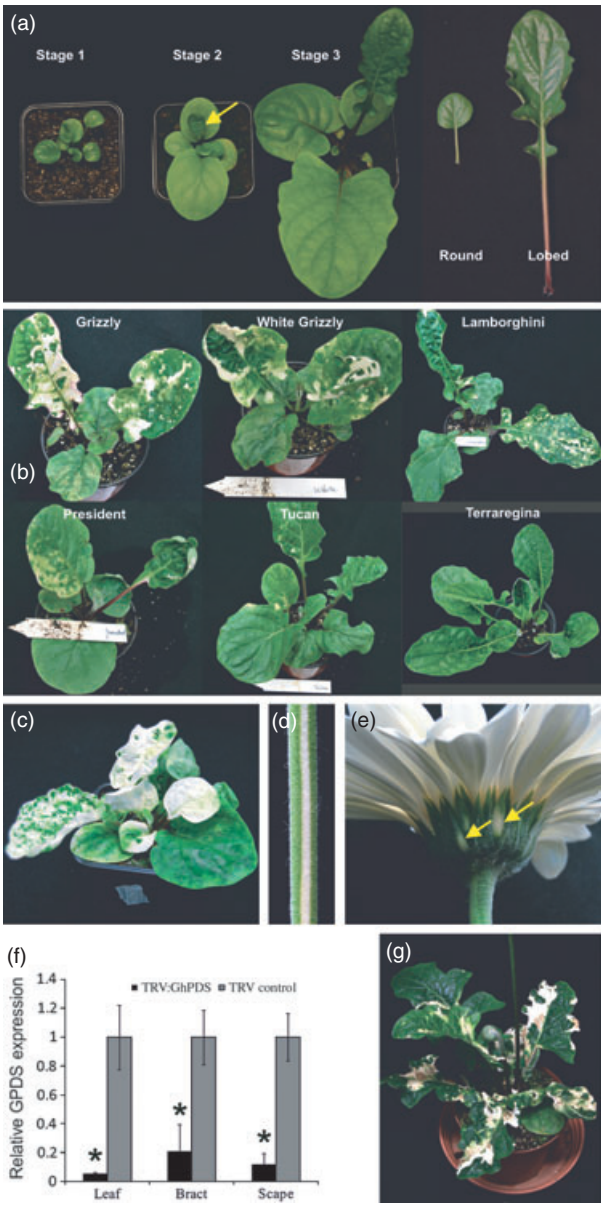

Figure 1 *Tobacco rattle virus* (TRV)-induced *PDS* silencing in gerbera. (a) Gerbera plantlets in three different developmental stages during vegetative growth. At stage 2, the leaf shape starts to turn from round to lobed (arrow). (b) Using syringe infiltration in gerbera leaves, five cultivars (Grizzly, White Grizzly, Lamborghini, President, and Tucan) of 21 showed *PDS* photobleaching symptoms. Cultivar Terraregina, along with 15 other cultivars, showed no silencing symptoms. (c) Vacuum-infiltrated plants from cultivar Terraregina showed intensive photobleaching phenotypes. (d, e) Vacuum-infiltrated gerbera plants from cultivar Grizzly and White Grizzly, respectively, showed intensive photobleaching symptoms on scapes (inflorescence stems) and involucral bracts (arrows). (f) *GPDS* transcript levels measured by QRT-PCR in cultivar Grizzly. Data are means ± SE (n = 3). (g) *GPDS*-silencing symptoms in a mature Grizzly plant (5 months old, 4 months after syringe infiltration).

plants showed photobleaching phenotypes while none of the Terraregina plant showed silencing symptoms. When syringe infiltrated at stage 3, only 1 of 12 inoculated Grizzly plants showed tiny photobleaching patches, and again no inoculated Terraregina plants showed silencing symptoms.

Vacuum infiltration, which has shown great efficiency in inducing gene silencing previously (Di Stilio *et al.*, 2010; Hileman *et al.*, 2005; Wang *et al.*, 2006; Ye *et al.*, 2009), was tested for cultivar Terraregina and for five other cultivars (Lamborghini, Grizzly, White Grizzly, President, and Tucan). Following vacuum infiltration at stage 2, all tested cultivars showed more intensive PDS-silencing symptoms than those inoculated with syringe infiltration at this growth stage. This also applied to the cultivar Terraregina (Figure 1c), which was hardly infected at all with syringe infiltration. The PDS-silencing symptoms continuously developed on newly developing leaves, scapes (inflorescence stem), and involucral bracts for more than 1 year in all cultivars (Figure 1d,e,g). The *GPDS* transcripts were reduced to 5.3%, 21%, and 12% of the normal levels in the photobleached leaves, involucral bracts, and scapes, respectively (Figure 1f, samples from cultivar White Grizzly were adopted for testing).

Silencing of two Mg-chelatase subunit genes in gerbera leaves

As the efficiency of target gene silencing by VIGS cannot be visibly assessed, concomitant silencing of a reporter gene is often used to visualize the tissue where silencing occurs. *PDS* is the most commonly used VIGS reporter, the silencing of which, however, significantly inhibits biosynthesis of carotenoids and GA, stability of chlorophyll, and many other metabolic pathways (Qin *et al.*, 2007). Therefore, in addition to *PDS*, we tested two other vegetative tissue reporters for gerbera.

Mg-chelatase is an enzyme involved in chlorophyll biosynthesis. It catalyzes the insertion of Mg^{2+} into protoporphyrin IX (Walker and Willows, 1997). Mg-chelatase subunits H and I have been used as VIGS reporters previously (Hiriart *et al.*, 2002; Igarashi *et al.*, 2009; Lu *et al.*, 2003). Their down-regulation leads to yellow patches on leaves where chlorophyll biosynthesis is interrupted but carotenoid biosynthesis is not affected. Fragments of gerbera Mg-chelatase subunit genes *GChl-H* and *GChl-I* (401 and 351 bp, respectively) were amplified from cultivar Terraregina to construct VIGS vectors TRV2:GChl-H and TRV2:GChl-I. These two VIGS constructs were tested with vacuum infiltration on cultivars Grizzly and Lamborghini at growth stage 2. Intensive silencing symptoms were observed in both cultivars. Several of the first developed leaves were white or yellowish (Figure 2a,b). Transcript levels of *GChl-H* and *GChl-I* in the silenced leaf tissues decreased to 6% and 20% of those in the controls, respectively (Figure 2c,d). The silencing symptoms continuously developed for about 1 month. After that, the newly emerging leaves appeared green. Intriguingly, TRV2:GChl-H induced symptoms appeared already at 2 weeks postinfiltration, which was 1 week earlier than that of TRV2:GChl-I. In parallel experiments, with syringe infiltration at stage 1, all 12 Lamborghini plants infected with TRV2:GChl-H developed clear silencing symptoms, but none of the plants inoculated with TRV2:GChl-I showed silencing phenotypes (data not shown). Thus, the TRV2:GChl-H construct used in this study turned out to be more efficient than TRV2:GChl-I for VIGS in gerbera.

Silencing of genes in gerbera inflorescences

One important aim in developing TRV VIGS system for gerbera is to study functions of genes involved in flower and inflorescence development. To test the efficiency of VIGS in floral tissues, and to develop a VIGS marker for flowers, a previously

Table 1 Responses of different gerbera cultivars on *Tobacco rattle virus* (TRV)-induced *PDS* silencing with syringe infiltration at stage 2 of vegetative development

Cultivar	Number of photobleached plants/number of treated plants*	Intensity of photobleaching†	Detection of TRV in photobleached tissue	Detection of TRV in non-photobleached, leaves‡
Terraregina	0/18	None	–	–
Grizzly	6/18	++++	6/6	3/6
White Grizzly	6/18	+++	6/6	3/6
President	12/18	+	12/12	3/12
Lamborghini	3/18	++	3/3	3/3
Tucan	9/18	+	9/9	0/9

*The numbers refer to total number of plants treated in three experimental replicates. †Intensity of photobleaching was visually estimated in each cultivar for the plant that showed most intensive silencing, as percentage of photobleached leaf area to total leaf area and scored with plus signs: +, <10%; ++, 10–20%; +++, 20–40%; ++++, 40–60%. ‡Sampled form plants showing photobleaching in lower leaves.

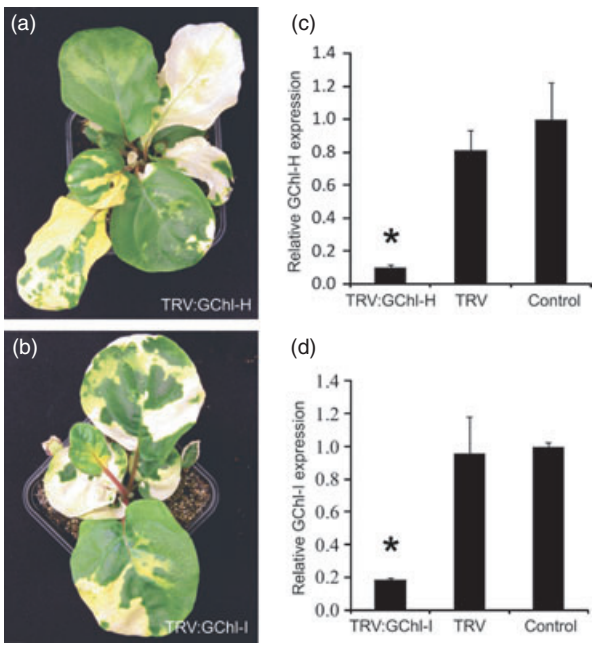

Figure 2 *Tobacco rattle virus* (TRV)-induced silencing of *GChl-H* and *GChl-I*, encoding Mg-chelatase H and I subunits in gerbera cultivar Lamborghini. (a, b) Symptoms on gerbera leaves after silencing of *GChl-H* and *GChl-I*. (c, d) QRT-PCR measured *GChl-H* and *GChl-I* transcript levels in silenced leaf, TRV-infected green leaf, and non-inoculated control leaf. Data are means ± SE (n = 3).

identified chalcone synthase encoding gene *GCHS1* of the anthocyanin pathway (Helariutta *et al.*, 1995) was used for testing. The VIGS vector TRV2:GCHS1 was developed by introducing of a 400-bp *GCHS1* gene fragment (bases 93–492 of the coding sequence) into pTRV2. Because fully grown and flowering gerbera plants were highly resistant upon syringe infiltration, and impractical for vacuum infiltration because of their large size, agrobacterium inocula carrying TRV VIGS vectors were applied directly on 2-cm-long scapes of developing inflorescence buds with scratch wounding. About 35% of the inoculated gerbera inflorescences in cultivars Terraregina and Lamborghini developed robust silencing phenotypes 3 weeks postinoculation, resembling those in stable *GCHS1* antisense transgenic lines (Elomaa *et al.*, 1993) (Figure 3a). The transcript level of *GCHS1* in gene-silenced petal tissues was decreased down to 2% of the control level (Figure 3d).

The gerbera MADS-box gene *GGLO1* (Yu *et al.*, 1999) was used to test whether VIGS could be used for flower developmental studies. *GGLO1* is a gerbera B-function gene that is involved in petal and stamen development (Broholm *et al.*, 2010; Yu *et al.*, 1999). Suppression of *GGLO1* expression by 35S-antisense construct in stably transformed lines converted the whorl two petals of the ray flowers into bract-like structures that contained chlorophyll (Yu *et al.*, 1999). In addition, in more severe lines, homeotic conversion of stamens in disc flowers into carpel-like structures was observed (Broholm *et al.*, 2010). The TRV vector, carrying a 400-bp *GGLO1* fragment (bases 140–539 of the coding sequence), was inoculated to 2-cm-long young scapes of plants from cultivar Grizzly. Three weeks after inoculation, six of 20 treated inflorescences in three of four treated plants developed typical *GGLO1*-silencing symptoms (Figure 3b), which were similar to those in *GGLO1* antisense transgenic plants (Yu *et al.*, 1999). About 30%–50% of petals of infected inflorescences developed as flat, bract-like organs, with reduced size and increased chlorophyll content in the abaxial sides (Figure 3b). The shape of the epidermal cells in the *GGLO1*-silenced bract-like organs was altered. They were broader, and the ridges were less pronounced compared with those of typical ray flower petal epidermal cells (Figure 3c). The transcript level of *GGLO1* in petals that showed silencing phenotypes was decreased to 20% of the control level (Figure 3e).

Expression of gerbera *2-pyrone synthase* was down-regulated by the silencing of reporter genes

One of our aims in developing VIGS for gerbera was to identify genes encoding enzymes for later steps of gerberin and parasorboside biosynthesis. The gerbera CHS-like polyketide synthase (G2PS1) is responsible for the first step in the pathway (Eckermann *et al.*, 1998), but to convert the 2PS product to gerberin and parasorboside, reduction and glucosylation is required. *G2PS1* is known to be highly expressed both in leaves and flower petals (Helariutta *et al.*, 1995). Surprisingly, quantitative real-time PCR (QRT-PCR) results showed that the expression of *G2PS1* was strongly down-regulated by silencing of *GPDS*, *GChl-H*, or *GChl-I* through VIGS. The transcript levels of *G2PS1* in the gene-silenced leaf tissues decreased to <5%

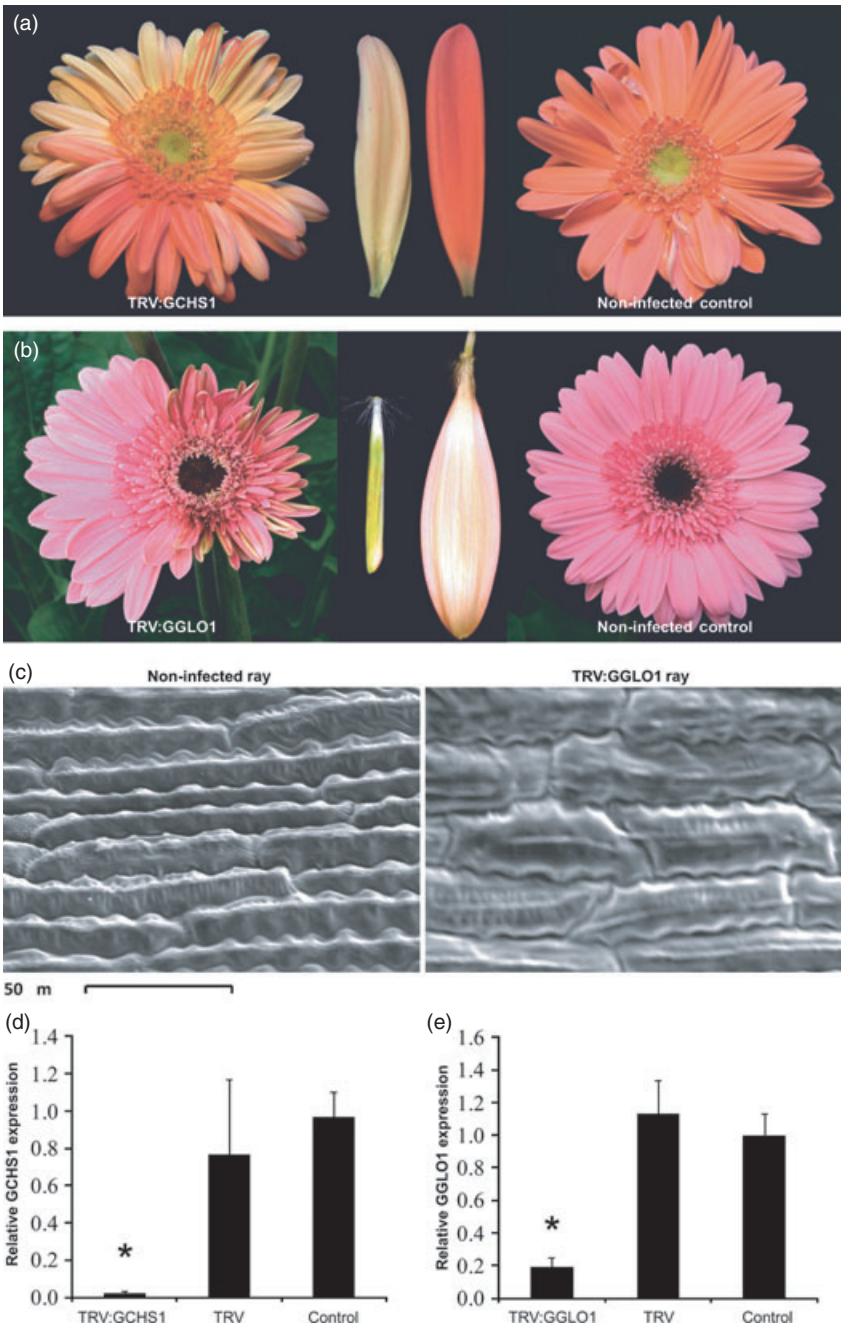

Figure 3 *Tobacco rattle virus* (TRV)-induced gene silencing in gerbera inflorescences. (a) TRV-induced *GCHS1*-silencing symptoms in gerbera inflorescences of cultivar Terraregina and the phenotypes of the adaxial sides of the petals (middle panel). (b) TRV-induced *GGLO1*-silencing symptoms in gerbera inflorescences of cultivar Grizzly and the phenotypes of the abaxial sides of the *GGLO1*-silenced whorl 2, bract-like organ, and the non-infected ray flower petal (middle panel). (c) Epidermal cell shapes of typical gerbera ray flower petals and gerbera *GGLO1*-silenced W2 organs detected by scanning electron microscopy. (d, e) QRT-PCR measured *GCHS1* transcript levels (samples from cultivar Terraregina, petal stage 8) and *GGLO1* (samples from cultivar Grizzly, petal stage 8). Data are means ± SE (n = 3).

compared with healthy leaves (Figure 4a,b). In non-infected plants (control) as well as in plants infected by TRV alone *G2PS1* expression varied (Figure 4a,b). However, *G2PS1* expression was consistent and strongly reduced by TRV:GPDS, TRV:GCHL-H and TRV:GCHL-I infection.

To further study the reason for *G2PS1* down-regulation, we developed photobleaching symptoms on gerbera leaves by treating the plants with norflurazon, a herbicide that functions through inhibition of PDS (Bartels and Watson, 1978). Gerbera plantlets, 3 weeks after potting, were treated with 20 μM of norflurazon in the growth point. Nine days after treatment, emerging leaves developed photobleaching symptoms (Figure 4c), which were visually similar to those observed in the *GPDS*-silenced leaves. QRT-PCR results showed that in the norflurazon-treated photobleached leaves, the transcripts of *G2PS1* were again strongly decreased to about 1% of levels in the non-treated leaves (Figure 4d). The transcript levels of *GPDS*, however, were not affected significantly (Figure 4d). It appears that photobleaching itself causes down-regulation of *G2PS1*.

Discussion

Virus-induced gene silencing technology has shown its potential as a reverse genetic tool, especially for plants with low transformation efficiencies and long life cycles (Sasaki *et al.*, 2011; Ye *et al.*, 2009), making, for example, studies on evolutionary development (evo-devo) much more efficient (Di Stilio, 2011). To date, there have been no reports of implementing VIGS in species of Asteraceae. In this study, we developed an efficient VIGS system for *Gerbera hybrida*. Through the selection of

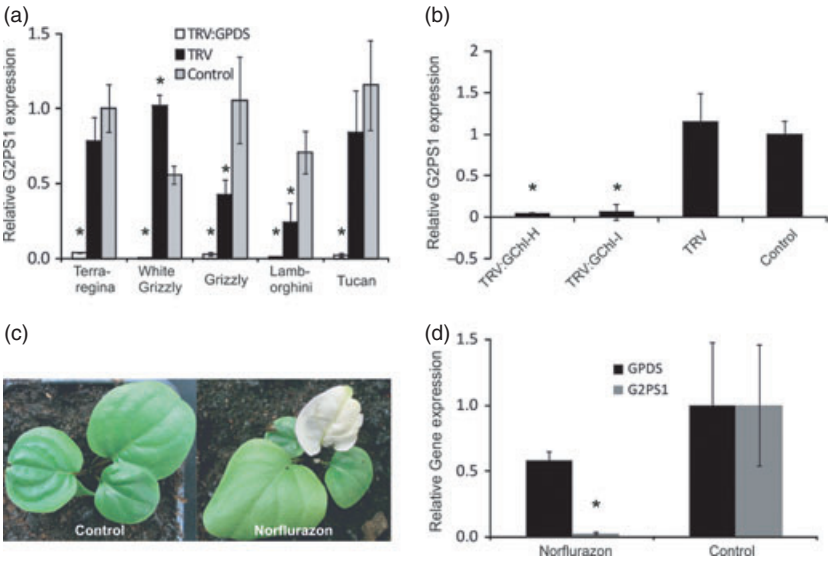

Figure 4 Gerbera *2PS1* expression was significantly down-regulated by the silencing of the gerbera *PDS*, *Chl-H*, and *Chl-I*. (a, b) QRT-PCR measured *G2PS1* transcript levels in leaf tissues of gene-silenced, *Tobacco rattle virus*-infected (green tissues), and non-treated controls. Samples in (b) were taken from gerbera plants of cultivar Lamborghini. (c) Norflurazon-induced photobleaching symptoms on gerbera plants of cultivar Terraregina. (d) QRT-PCR measured *G2PS1* and *GPDS* transcript levels in leaf tissues showing photobleaching induced by norflurazon and non-treated control. Data are means ± SE (n = 3).

TRV-sensitive cultivars and improving the inoculation methods, we succeeded in silencing three different marker genes (*GPDS*, *GChl-H*, and *GChl-I*) in leaf tissues. In addition, we obtained robust gene silencing in floral tissues with VIGS by targeting genes responsible for anthocyanin biosynthesis (*GCHS1*) (Elomaa *et al.*, 1993) and flower organ development (*GGLO1*) (Broholm *et al.*, 2010; Yu *et al.*, 1999).

Genetic differences among the cultivars of the same species may result in differences in their susceptibility to TRV infection (Ghazala and Varrelmann, 2007; MacFarlane, 2010) or TRV VIGS (Chen *et al.*, 2004). We also observed that different gerbera cultivars showed large variation in their TRV VIGS responses. Cultivar Terraregina, routinely used for stable transformation and large-scale sequencing in our laboratory, showed practically no silencing phenotypes following syringe agroinfiltration of the VIGS vector. However, five of the other 21 tested cultivars showed photobleaching symptoms, starting from 2 weeks postinfiltration. Except for cultivar President that displayed severe disease symptoms by the infection of TRV (Figure S1), the other four cultivars showed almost no disease symptoms. Cultivar Grizzly had the best performance upon VIGS-based silencing. The silencing phenotype developed early was most intensive, and importantly, no disease symptoms were induced. In addition, our results showed that gerbera plantlets at stage 1 of vegetative development were much more sensitive to TRV VIGS than those at stage 2 or 3.

Previously, vacuum infiltration has shown its great efficiency in inducing gene silencing (Di Stilio *et al.*, 2010; Hileman *et al.*, 2005; Wang *et al.*, 2006; Ye *et al.*, 2009). Compared with syringe infiltration, the vacuum infiltration method induced more intensive silencing in gerbera, and all six treated cultivars including Terraregina showed PDS-silencing symptoms. However, there are also some disadvantages associated with vacuum infiltration, including the need to remove the plantlets from soil or growth medium and the damage to the plantlets by vacuum. In our experiments, about 20% of plantlets were severely injured by the vacuum treatment. It also slowed down the development of the plants, which extended the time to observe VIGS phenotypes.

For VIGS on gerbera inflorescences, we applied the agrobacterium constructs on scapes of the newly developing inflorescence buds using mechanical wounding. We obtained silencing of an anthocyanin pathway gene *GCHS1* and a MADS-box gene *GGLO1* and confirmed the phenotypes previously observed by stable transformation of gerbera with the corresponding antisense constructs (Elomaa *et al.*, 1993; Yu *et al.*, 1999).

Owing to the irregular distribution of the VIGS vector in the host plants and systemically infected leaves, virus-induced gene silencing in target tissues is usually patchy (Quadrana *et al.*, 2011). Thus, it is necessary to couple VIGS with an easily traceable silencing marker gene, particularly when silencing of the target gene does not give any visible phenotype. For gerbera, genes encoding phytoene desaturase and subunits of Mg-chelatase are useful markers for the vegetative tissues, and *GCHS1* for pigmented floral tissues. Silencing of those marker genes gave clearly visible phenotypes in our experiments. However, suitable marker genes for VIGS should be carefully selected. Proper experiments need to be carried out for the evaluation of any possible impact of the marker gene silencing on unintended target gene expression.

Using *G2PS1* as an example, we showed that silencing of *GPDS* and genes for Mg-chelatase subunits dramatically down-regulated an unrelated gene. This was unexpected because PDS is involved in carotenoid synthesis, and Mg-chelatase in chlorophyll synthesis, and neither of them has any obvious connection with the 2PS catalyzed gerberin and parasorboside pathway (Eckermann *et al.*, 1998). We also tested the effect of TRV infection itself on *G2PS1* expression using the empty TRV vector. *G2PS1* expression levels varied both in mock-infected plants and non-infected control plants. However, *G2PS1* expression was never as dramatically low as in the plants infected by the TRV: reporter constructs. Therefore, we postulate that the virus itself is not affecting *G2PS1* expression. Furthermore, chemical inhibition of GPDS by the herbicide norflurazon (Bartels and Watson, 1978) not only produced carotenoid and chlorophyll deficient white leaves on gerbera, but also concomitantly abolished *G2PS1* mRNA accumulation. To proceed in the analysis of the gerberin/parasoboside pathway, a suitable VIGS reporter is yet to be identified. Gerberin and parasorboside are synthesized also in floral tissues (Koskela *et al.*, 2011), and from this point-of-view, *GCHS1* could be used for VIGS, but as it is highly similar to *G2PS1*, cross down-regulation may be another obstacle.

In conclusion, we have shown that TRV VIGS can be implemented for assessing gene functions in gerbera. The optimized TRV VIGS system can be used to target the silencing of genes both in gerbera leaves and inflorescences. Importantly, TRV VIGS is also promising for silencing genes affecting flower development. Expanding the use of VIGS to gerbera is valuable for functional genomics research on species of the whole sunflower family and will further facilitate comparative studies to better understand the evolution of developmental and metabolic diversity. Our results also indicate that the selection of marker genes for VIGS must be made with care. Strict evaluation needs to be performed to avoid any misinterpretations.

Experimental procedures

Plant material

All gerbera cultivars were obtained from Terra Nigra B.V., the Netherlands. Gerbera plants were multiplied and rooted *in vitro* as previously described (Elomaa and Teeri, 2001). The rooted gerbera plantlets were then potted in soil (mixture of peat and vermiculite in volume ratio of 1 : 1), and grown in the growth room in controlled conditions: day/night temperature 21/19 °C; photoperiod 16 h; light intensity 250 μmol m^{-2} s^{-1}. Two weeks after potting in soil, gerbera plants were moved into the greenhouse. The day/night temperature in the greenhouse was 18–20/16–18 °C. The day length followed natural day length in the summer time and was extended to 10 h in the winter. Gerbera inflorescence developmental stages have been described previously (Helariutta *et al.*, 1993).

Construction of VIGS vectors

The coding sequences of *GCHS1* (GenBank: Z38096.1), *G2PS1* (GenBank: Z38097.2), and *GGLO1* (GenBank: AJ009726.1) have been previously published. Sequences of *GPDS*, *GChl-H*, and *GChl-I* were identified from a gerbera EST database (Laitinen *et al.*, 2005) using *Arabidopsis* and tobacco sequences as queries in blast searches. Fragments of about 400 bp for each transcript were PCR amplified with gene-specific primers (Table S2) from cDNA of leaf tissues (for *GPDS*, *GChl-H*, and *GChl-I*) or petals (for *GCHS1* and *GGLO1*) of cultivar Terraregina. These fragments were subsequently cloned into TRV2 Gateway vectors (Liu *et al.*, 2002) supplied by Dr. Dinesh-Kumar (Yale University). Nucleotide sequences of the *GPDS*, *GChl-H*, and *GChl-I* fragments were submitted to gene bank under the accession numbers JQ894780, JQ894781, and JQ894782, respectively.

Agrobacterium infiltration

Virus-induced gene silencing vectors carrying target gene fragments, as well as pTRV1 and pTRV2 (Liu *et al.*, 2002), were transformed into *Agrobacterium tumefaciens* strain C58C1(pGV2260) (Deblaere *et al.*, 1985) by electroporation. On the basis of our previous studies, this strain has turned out to be the most efficient for gerbera, at least in stable transformation (Paula Elomaa, unpublished). Agrobacterium cells were grown and collected as previously described (Liu *et al.*, 2002). The harvested bacterium cells were then resuspended to OD_{600} of 2 in infiltration medium containing 10 mM MES, 200 μM acetosyringone, and 10 mM $MgCl_2$ and incubated at room temperature for 3 h. Before infiltration, bacteria carrying pTRV1 and pTRV2 (or pTRV2 derivates) were mixed in 1 : 1 volume ratio. For syringe infiltration, agrobacterium inocula were introduced into two lower leaves from the abaxial side with a 1 ml syringe without a needle. For vacuum infiltration, whole plantlets were removed from *in vitro* rooting medium and were submerged into agrobacterium suspension and subjected to vacuum. When air bubbling from plantlets started to decrease, the vacuum was released quickly to allow bacteria to enter the plant tissues. The time for the vacuum treatment varied from 30 s up to 3 min depending on the vacuum source we used. After vacuum infiltration, plantlets were rinsed with water, potted in soil, and moved to growth rooms. For silencing genes in the inflorescences, scapes (inflorescence stems) of about 2 cm in length were wounded by scratching, and agrobacterium inocula (about 1 ml for each scape) was introduced in the surface of wounded area by covering the wound with agrobacterium-wetted cotton pellets.

For screening of gerbera cultivars, six plantlets of each cultivar were syringe infiltrated at stage 2 of vegetative development. The gerbera *PDS* gene was used as a silencing marker. *GPDS*-silencing intensities were visually scored as percentages of photobleached leaf area of the whole leaf area. For vacuum infiltration, 12 plantlets of each selected cultivar were treated. For silencing genes in gerbera inflorescences, about 20 scapes, from six plants, were inoculated. Cultivars Terraregina and Lamborghini were used for silencing of *GCHS1*, and cultivar Grizzly was used for silencing of *GGLO1*. All experiments were repeated three times.

RNA analysis

To analyze the effect of VIGS on target gene expression, samples were collected from tissue areas showing the silencing phenotypes. For control, corresponding samples were collected from tissues infected by TRV carrying no host gene fragment insert or from non-infected plants tissues. For each treatment, samples from three independent biological replicates were analyzed. Total RNA of gerbera leaf and petal tissues (stage 8, Helariutta *et al.*, 1993) was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. DNase (Promega) treatment was performed to remove any genomic DNA residues. The first-strand cDNA was synthesized from 1 μg of total RNA using SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). The synthesized 20 μl of cDNA was then diluted with Milli-Q water to the final volume of 200 μl.

For the detection of the presence of TRV RNA in the infected tissue, RT-PCR was carried out using TRV RNA1– and RNA2-specific primers (Table S2). The conditions for RT-PRC were as follows: one cycle for denaturation (94 °C, 3 min) followed by 30 cycles for amplification (94 °C, 30 s, 60 °C, 30 s, 72 °C, 1 min). DyNAzyme II DNA-polymerase (Thermo Scientific Finnzymes, Vantaa, Finland) was used. The expression of target genes was verified by QRT-PCR. QRT-PCR was carried out with the Light Cycler® 480 Real-Time PCR detection system (Roche, Basel, Switzerland). The PCR Master Mix (15 μl) contained 4.5 μl cDNA template, 7.5 μl of LightCycler 480 SYBR Green I Master, and 1.5 μl (5 μM) gene-specific primers (Table S2). The thermal programme was as follows: 1 initial cycle of denaturation (95 °C for 10 min), followed by 45 cycles of amplification (denaturation 95 °C, 10 s; annealing 62 °C, 10 s; elongation and signal acquisition, 72 °C, 10 s). The relative expression levels were calculated with the $\Delta\Delta C_t$ method (Pfaffl, 2001). For each biological replicate, three technical replicates were run. We tested three different candidate reference genes encoding gerbera ACTIN,

UBIQUITIN, and GAPDH, of which the ACTIN-encoding gene was used to normalize the expression levels. In all figures, the data are shown as an average expression value in the three biological replicates relative to that in the control sample that was set as one. The error bars indicate the standard error (SE) calculated from the three biological replicates. One-way ANOVA was performed to analyze the significant differences in gene expression between each treatment, and the control and is indicated with *($P \leq 0.05$) in figures.

Scanning electron microscopy (SEM)

Gerbera samples were fixed in fresh FAA buffer (50% ethanol, 5% acetic acid, and 2% formaldehyde) overnight and then dehydrated through ethanol series (50%, 60%, 70%, 80%, 95%, and twice in 100%; treat 30–60 min each time). After critical point drying (Balzers CPD 020 Bal-Tec, Balzers, Liechtenstein), samples were mounted on aluminium stubs and coated with platinum/palladium. Specimens were scanned with electron microscope (Zeiss DSM 962, Zeiss, Oberkochen, Germany) at the Institute of Biotechnology Electron Microscopy Laboratory, University of Helsinki.

Norflurazon treatment

Gerbera plantlets of cultivar Terraregina, 3 weeks after potting in soil, were used for norflurazon treatment. A stock (5 mM, 250×) of norflurazon (Sigma-Aldrich, Saint Louis, MO) was made in 100% ethanol. The stock was diluted in Milli-Q water to the concentration of 20 μM before treatment. Norflurazon solution was applied to newly developed leaves with a small acrylic paint brush once a day continuously for 8 days.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 *Tobacco rattle virus*-induced symptoms in gerbera cultivar President.

Figure S2 Detection of TRV RNAs in TRV-infected and gene-silenced plant tissues by RT-PCR.

Table S1 *Gerbera hybrida* cultivars used for TRV VIGS screen.

Table S2 Primers used for constructing VIGS vectors and QRT-PCR.

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