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Tobacco rattle virus-based virus-induced gene silencing (VIGS) as an aid for functional genomics in Saffron (Crocus sativus L.)

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Abstract

Several limitations in genetic engineering interventions in saffron exist, hindering the development of genetically modified varieties and the widespread application of genetic engineering in this crop. Lack of genome sequence information, complexity of genetic makeup and lack of well-established genetic transformation protocols limits its *in planta* functional validation of genes that would eventually lead toward crop optimization. In this study, we demonstrate agro infiltration in leaves of adult plants and whole corm before sprouting are suitable for transient gene silencing in saffron using Tobacco Rattle Virus (TRV) based virus-induced gene silencing (VIGS) targeting phytoene desaturase (PDS). Silencing of PDS resulted in bleached phenotype in leaves in both methods. TRV-mediated VIGS could be attained in saffron leaves and corms, providing an opportunity for functional genomics studies in this expensive spice crop.

Introduction

Saffron (*Crocus sativus* L.) is a geophytic plant belonging to the family Iridaceae. Saffron is a plant with economic significance, not only as a culinary spice but also due to its pharmacological potential (Gohari et al. 2013). The sterile nature of saffron poses a challenge to traditional breeding methods that rely on the exchange of genetic material through sexual reproduction. Saffron plants primarily propagate vegetatively through corms, which are underground storage organs. The limited genetic diversity adds to the ability to introduce desired traits through conventional breeding. In such cases, biotechnological approaches become valuable tools for genetic improvement. A comprehensive understanding of the genetic and molecular mechanisms controlling different developmental pathways can aid biotechnological approaches in saffron genetic improvement. In recent years, transcriptomic studies in saffron have identified numerous genes and transcription factors, but the lack of an efficient genetic transformation system impedes the progression to conclusive functional studies. Agrobacterium-mediated transformation is widely used to manipulate genes in a variety of dicots but with limitations in monocots. Saffron is a monocot and to date, no efficient protocols are available for its genetic transformation. Thus, there is a need to develop alternative methods for functional validation of genes.

Virus-induced gene silencing (VIGS) is a valuable tool in plant molecular biology that offers flexibility in studying gene functions across various plant species (Burch-Smith, Miller et al. 2006). VIGS implies evolutionarily conserved antiviral defence mechanisms present in plants (Baulcombe 2004). In general, the target gene of interest is integrated into a viral vector system. The viral vector acts as a delivery vehicle, carrying the genetic material that triggers the post-translational gene silencing mechanism against the target gene (Vance and Vaucheret 2001). VIGS enables the analysis of gene functions in plant species that are not easily amenable to stable genetic transformation. VIGS has proven to be a valuable tool for examining gene functions in various plant species, including monocot crops like rice (Kant and Dasgupta 2017), maize, wheat (Zhang et al. 2017), and barley (Yuan et al. 2011). VIGS has also been successfully employed for the functional characterization of genes in monocot geophytes such as Gladiolus (Li et al. 2023), Tulips (Sun et al. 2022), Lilium (Xu et al. 2019; Pan et al. 2023).

The availability of advanced VIGS vectors with broad host ranges and high silencing efficiency has significantly facilitated gene function studies in monocot plants (Mei and Whitham 2018; Mei et al. 2016; Ding et al. 2006; Holzberg et al. 2002). For example Tobacco rattle virus (TRV)(Ratcliff et al. 2001), foxtail mosaic virus (FoMV)(Liu et al. 2016), Potato virus X (PVX)(Jones et al. 1999), Barley stripe mosaic virus (BSMV)(Holzberg et al. 2002), Tobacco mosaic virus (TMV) (Kumagai et al. 1995), Cabbage leaf curl virus (CaLCuV) (Muangsan et al. 2004). However, the Tobacco rattle virus (TRV) vector stands out as a valuable tool in plant molecular biology and functional genomics. Its ability to induce efficient gene silencing without causing overt viral symptoms or significant negative effects on host plant growth has made it a preferred choice for researchers studying gene functions in diverse plant species.

In this study, we examined the use of VIGS in saffron via silencing Phytoene Desaturase (PDS), a gene widely used as a marker due to its visible phenotype in the VIGS system (Cunningham Jr and Gantt 1998). PDS encodes for a carotenoid biosynthesis gene and silencing it blocks the production of carotenoids and causes the degradation of chlorophyll, leading to visible photobleached phenotype (Di Stilio et al. 2010). VIGS has not been established nor reported in saffron. Our study provides the first successful demonstration of the Virus-Induced Gene Silencing (VIGS) system in inducing endogenous gene silencing in saffron. The successful use of VIGS lays the foundation for future studies aiming to characterize gene function in saffron.

Materials and methods

Plant materials

Saffron corms of 8 to 10g size were used for the study. Corms used in the study were grown and harvested from fields of CSIR-IHBT, Palampur, India. Corms were planted in October, in growth chambers at 22°C Day/18°C Night and 16 h Light/8 h Dark Cycles, and watered at weekly intervals. For leaf infiltration experiments, corms were planted in October 2022, in growth chambers at 22°C Day/18°C Night and 16 h Light/8 h Dark Cycles, and watered at weekly intervals. Leaves of three-month-old plants were infiltrated. For whole corm infiltration experiments, freshly harvested corms from the 2023 harvest were used. Agro infiltration was performed twice using the vacuum method during July 2023 (Dormant stage) and September 2023 (Sprouting stage). After 1st vacuum infiltration corms were incubated at 22°C Dark, and after 2nd vacuum infiltration, corms were planted and shifted to growth chambers at 22°C Day/18°C Night and 16 h Light/8 h Dark Cycles, and watered at weekly intervals.

Gene Identification and Construct Preparation

The target gene PDS sequence was obtained from NCBI (GenBank: AY183118.1). Nucleotide sequences were aligned with other PDS sequences to identify highly conserved stretches of nucleotides for VIGS (Figure S1). A 405-bp conserved fragment of *CsatPDS* was amplified using the forward CsPDSF xba1 and CsPDSR sac1 reverse primer and subsequently cloned into the pJET cloning vector. The TRV2 and pJET-CsatPDS plasmids were digested with XbaI and SacI, purified and ligated using T4 DNA ligase enzyme, and transformed into E. coli DH5alpha. Colonies were selected on LB plates containing 50 mg/L

of kanamycin, and the presence of insert was confirmed by PCR with primers spanning the Multiple Cloning Sites of TRV2. The expected size of the PCR product was 100 bp in the empty TRV2 vector, and the resulting amplicon size was 505 bp in the presence of the *CsatPDS* insert. The plasmid was further sequenced to verify the correct insertion of the fragment.

Agroinfiltration of Crocus sativus

Transformation of A. tumefaciens with the generated constructs was performed. Here, competent A. tumefaciens GV3101 cells were transformed via flash freeze and tested for the presence of the targeted inserts. Transformed A. tumefaciens were streaked on LB agar plates containing 50 µg/ml kanamycin, rifampicin and, gentamicin and incubated at 28°C for 2 days. A single colony of A. tumefaciens was used to inoculate 5 ml LB liquid medium with the mentioned antibiotics and incubated at 28°C for 24 h at 180 rpm, after which 5 ml LB culture was used to inoculate 50 ml of LB medium with the antibiotics and subsequent incubation at the above conditions. Transformed cells were pelleted by centrifugation at 6080 rpm for 15 min and resuspended twice in 50 ml of 10 mM MgCl2. Fresh infiltration solution was used to resuspend the cells, followed by incubation at 28°C for 2–3 h at 200 rpm at which time the culture was diluted to an OD600nm of 1.2 with infiltration medium (prepare fresh before infiltration: 10 mM MgCl2, 10 mM 2-(N-morpholino) ethane sulfonic acid (MES), and 200 µm acetosyringone). Finally, 5 µl of Silwet L-77 was added.

For agroinfiltration, both the abaxial and adaxial sides of leaves were infiltrated per plant by piercing the leaf with a 1ml needle syringe and placed in a growth chamber at 22°C without light for 48 hrs and then to growth condition 16L/8D 20°C. For whole corm infection, infiltration solutions were scaled up to submerge the corms and provided a vacuum for 20 minutes using a Millipore vacuum pump (25Kpa) twice before sprouting. Vacuum-infiltrated corms were incubated in the dark at 22°C for 48 hours and transferred to optimum growth conditions till sprouting.

Confirmation of infection and Validation

To confirm the systemic virus infection of the Saffron plants, leaf tissue was observed under a microscope (Magnus TM) and samples were harvested for RT-PCR, sequence verification, and stored at − 80°C until further analysis. Subsequently, the tissue was ground to a fine powder with autoclaved mortars and pestles for RNA extractions. The total RNA was extracted using a Plant Total RNA kit (Sigma-Aldrich). The initial quality of RNA was determined by NanoDrop™ one/oneC microvolume UV-vis spectrophotometer (Thermo Fisher Scientific). The RNA quality was checked by electrophoresis on a 1.0% (w/v) agarose gel. The cDNA was synthesized by the Verso reverse transcriptase cDNA synthesis kit. For PCR amplification, a protocol was as follows: initial denaturation at 95°C for 3 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30s, and extension at 72°C for 1min s; completed by final elongation. Amplicons were analysed via Sanger sequencing.

Results

Isolation, sequence analysis and construct preparation of the PDS gene from Crocus sativus for VIGS

The multiple sequence alignment of saffron PDS with PDS from other plants suggested that they share high sequence similarity (Supplementary Fig. 1A) and are a good candidate as a marker gene to study gene silencing by VIGS. Primers were designed bordering the conserved region to isolate PDS gene fragments to be used for the VIGS study. The PDS gene fragment was amplified from *Crocus sativus* cDNA with forward and reverse primers (Supplementary Table 1). An amplification of approximately 400bp fragment was obtained, cloned and sequenced in the TRV2 vector (Supplementary Fig. 1b). The TRV2 vector had fragment sizes of 100bp with vector-specific primers and that of TRV2- *CsatPDS* plasmid was approximately 500bp, providing that the 405bp conserved fragment was inserted into the TRV2 vector (Supplementary Fig. 1c). Further results were validated by sequencing and the cloned sequence showed 100% alignment with the PDS gene. The above-mentioned outcomes suggested that we successfully constructed the TRV2-*CsatPDS* vector (Supplementary Fig. 1d).

Silencing of PDS gene in saffron by leaf infiltration

Saffron plants were inoculated with Agrobacterium cultures containing TRV1 + TRV2 (mock), and TRV1 + TRV2:*CsatPDS* (Treated) and examined for phenotypic changes compared with untreated plants (Fig. 1a). Constructs were delivered into leaves by injection/infiltration using a syringe. Out of the 6 plants (With multiple leaves) that were infected with *CsatPDS* (TRV2) vectors, only 4 plants showed signs of photobleaching after 1 week of infiltration, probably due to silencing of PDS (Fig. 1b). To determine whether the TRV-based vector can be transmitted, treated leaves were tested for the presence of the PDS insert. The PDS insert still appeared in all of the samples. Detection of TRV1 and TRV2-CsPDS transcripts in mock-treated and VIGS-treated leaves provided evidence that silencing was due to the TRV-based vector treatment. Viral expression was present in mock-treated and TRV2:*CsatPDS*-treated groups (Fig. 1c). However, TRV1 or TRV2 transcripts were not detected in untreated plants. Further, to confirm the *CsatPDS* silencing at the molecular level, we performed quantitative real-time RT-PCR on the treated leaves exhibiting a silencing phenotype compared with untreated and mock-treated saffron leaves. There was a significant down-regulation of *CsatPDS* in photobleached leaf samples, though endogenous PDS gene expression in mock-treated leaves did not decrease significantly compared to the untreated leaves (Fig. 1d). Results indicate that the *CsatPDS* gene in saffron plants was effectively silenced and TRV infection was systemically established. However, the degree of silencing for these 4 (33.3%) plants was so strong that two of the plants dried shortly after the photobleaching phenotype was observed, most likely as a result of excessive photobleaching caused by VIGS of *CsatPDS*. The leaves of untreated and mock-treated plants still remained green during this period.

Silencing of PDS gene in corm tissue by vacuum infiltration

Following the observation of effective silencing of the PDS gene using VIGS in saffron leaves, we further investigated the effectiveness of the TRV-based silencing of endogenous genes in the saffron corm. Dormant saffron corms were infiltrated with TRV2:*CsatPDS* silencing and mock constructs following their growth in optimum conditions (Fig. 2a). The primary leaves of saffron plants were examined for

photobleaching phenotype. In the corm, once sprouting began, the first newly developed leaves began displaying a silencing phenotype eight weeks after vacuum infiltration. Among the 20 TRV1 + TRV2:*CsatPDS* treated plants, 30% exhibited blotchy leaves due to *CsatPDS* silencing. On the other hand, 50% of treated plants show completely bleached leaves after sprouting (Fig. 2b). we checked for integration and expression of TRV vectors in the corm and newly emerging leaf tissues. Both the tissues showed integration of empty (mock-treated) and TRV2:*CsatPDS* in treated samples suggesting their successful expression. The identification of TRV1 and TRV2:*CsatPDS* transcripts in both mock-treated and ViGS-treated leaves implies that the observed silencing can be attributed to the TRV-based vector treatment. Viral expression was evident in both the mock-treated and TRV2:*CsatPDS*-treated groups (Fig. 2c). Results reveal effective silencing of the PDS gene in saffron plants, with the systemic establishment of TRV infection. Throughout this period, the leaves of both untreated and mock-treated saffron plants retained their characteristic green colour, serving as reference points for comparison (Fig. 2b).

Secondly, we utilized RT-qPCR and checked the silencing of the PDS gene in corm and leaf tissues. Comparative analysis of relative gene expression was conducted against control (untreated) plants those treated with empty vector (mock-treated) and those treated with PDS gene (Treated). There was a notable and statistically significant decrease in gene expression in treated corms and newly developed leaves when compared to those treated with the empty TRV1 + TRV2 (Mock treated) vector and control plants (Fig. 2d and e).

Discussion

Saffron is an economically important perennial spicy herb (Iridaceae family) and is well known as ‘Red Gold’ in producer countries. It has been cultivated for its stigmas, which not only comprise a highly valued spice but also have various therapeutic uses. The sterile nature and vegetative mode of reproduction of the species renders improvement by conventional breeding very difficult. Biotechnological approaches such as genome editing, cisgenics etc., can provide solutions for developing saffron varieties with desired traits. Although a poor understanding of molecular mechanisms regulating various developmental and metabolic processes is a bottleneck for the above. Recent advancement in omics technologies has led to the identification of several genes and transcription factors involved in various plant developmental processes, but due to the lack of an efficient genetic transformation system, their functional validation is lacking in saffron (Kalia et al. 2022; Jose-Santhi et al. 2023; Bagri et al. 2017; Hu et al. 2020; Renau-Morata et al. 2021). Due to this limitation, no functional genomic studies have been performed in saffron. Thus there is an unmet need to develop alternative and efficient methods for functional validation of gene function in saffron.

The present study aimed to investigate the efficacy of Tobacco Rattle Virus (TRV)-based vectors as inducers for gene silencing in saffron. The goal was to explore whether TRV-based vectors could serve as a practical and effective method for silencing endogenous genes in saffron plants. Successful gene silencing could pave the way for a faster and more efficient method for characterizing the functions of

genes in saffron. VIGS has played a critical role in gene function studies (Senthil-Kumar and Mysore 2014). Additionally, VIGS has been widely used in various plant species as an efficient and powerful gene-silencing technique (Dommes et al. 2018; Au - Velásquez et al. 2009; Wege et al. 2007). Our results showed that VIGS could be performed in saffron with infiltration in leaves as well as in corms. PDS gene silencing was obtained successfully which was capable of causing a visible photobleached phenotype in both developed and newly developing leaves. The photobleaching phenotype observed in *CsatPDS*-silenced leaves might have resulted from impaired biosynthesis of carotenoids (Ratcliff et al. 2001; Liu et al. 2016). Additionally, TRV could also infect corm tissue suggesting that the method can be used for silencing of genes expressed in corms and related to corm function. The detection of phenotype after 8 weeks of infection suggests that this method can be applied to study gene function involved in longer plant developmental processes. TRV-based VIGS system has already been reported for functional validation of genes in monocot geophytes, where a stable transformation system is not established. Recently, with TRV-based VIGS as a tool, several important findings focusing on the molecular aspects of bulb development, dormancy release, and bud growth have been concluded in Tulips, Gladiolus and Lilium (Sun (Sun et al. 2022; Li et al. 2023; Pan et al. 2023). This suggests that the VIGS system is an efficient, reliable and quick method for functional gene studies concerning plant development and metabolism. Thus, VIGS might be employed to characterize genes regulating important traits in saffron. This work provides a strong foundation for further studies that could lead to the quick characterization of thousands of genes identified for their putative role in different developmental processes in saffron.

Conclusion

All together the data indicate that the TRV-based VIGS system can be used for functional studies of genes in saffron. Our findings showed that quick and effective VIGS was established in C. sativus. The approach described above provides an innovative approach for developing a VIGS mechanism, which raises the possibility of high-throughput *in planta* functional gene validation research in saffron.

Declarations

Acknowledgements

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Author contribution statement

R.K.S conceived the research plan; D.K, J.J.S, F.R.S, D.S, and R.K.S performed the experiments; D.K, J.J.S & R.K.S analyzed the data and wrote the article. All Authors read the article and approved the final manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

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Supplementary Files

Supplementary Figures and Tables is not available with this version.

Figures

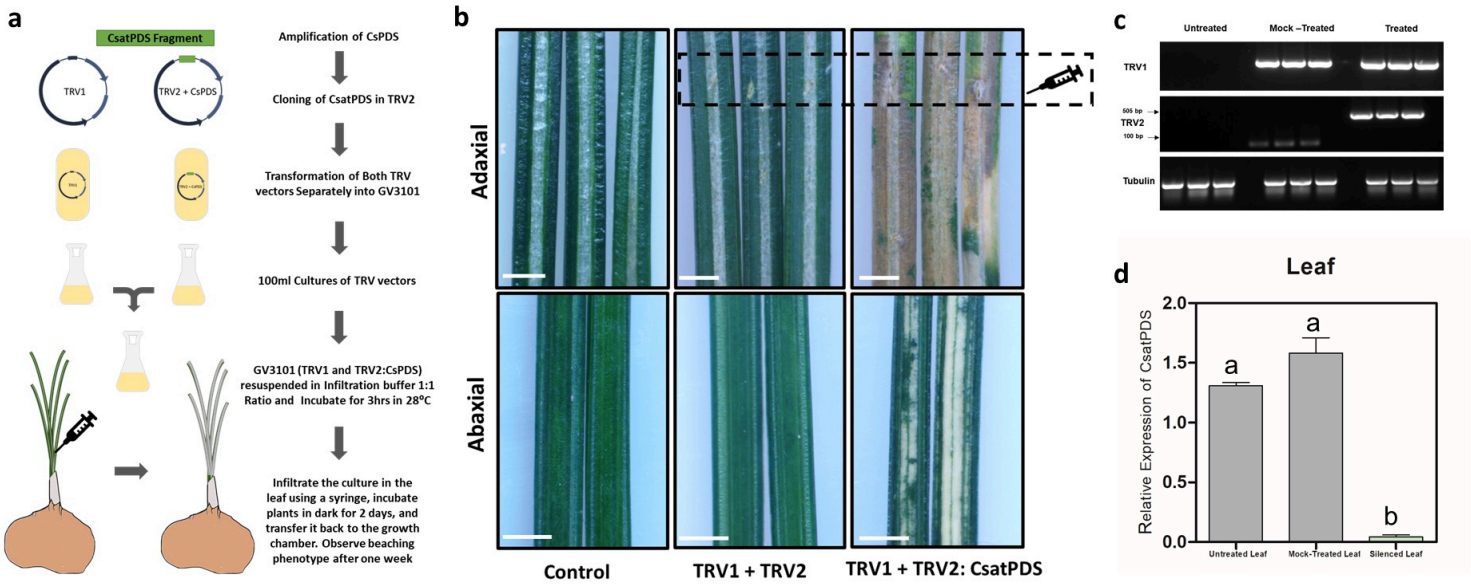


Figure 1

VIGS experiment targeting *CsatPDS* in *Crocus sativus* leaves. (a) The schematic representation of workflow used for the study. It involves the construction of a vector system, the transformation of constructs into Agrobacterium strain GV3101, and the infiltration of culture in the leaves using a syringe. Bleaching phenotype observed among leaves after one-week post-inoculation. (b) Phenotypic observation of bleached leaves under the microscope of both abaxial and adaxial sides. The scale bar represents 3mm. The experiment group involves control (untreated crocus sativus plants, TRV1+TRV2 (Mock Treated), and TRV1+TRV2:*CsatPDS*(Treated). Treated plants (TRV1+TRV2:*CsatPDS*) show bleaching phenotype at both the adaxial and abaxial surfaces of a leaf. Area under doted box represent

area of syringe insertion (c) Detection of TRV transcript by RT-PCR in VIGS photobleached leaves of *Crocus sativus* after vacuum infiltration. Three groups were considered: untreated plants, those treated with empty TRV2 vector as a control (mock-treated), and plants treated with TRV2-*CsatPDS*, carrying the *CsatPDS* insert. RT-PCR was conducted using specific primers for the housekeeping gene *CsatTubulin* (Used as an internal control) and viral transcripts TRV1/TRV2. The TRV2 vector displayed two bands: a larger band (505bp) resulting from the *CsatPDS* insert (405bp), and a smaller band (100bp) from an empty TRV2 (mock-treated). The TRV1 vector shows a single band of 500bp in treated and mock-treated corms. (d) Quantitative Real-Time PCR expression profiling of *CsatPDS* in control, mock and treated leaves showing reduction of PDS transcript after VIGS. Expression values of the *CsatPDS* gene are averages for three biological replicates ± SEM, relative to the reference gene tubulin. Different letters a–b over the bars indicate significant differences at P < 0.005.

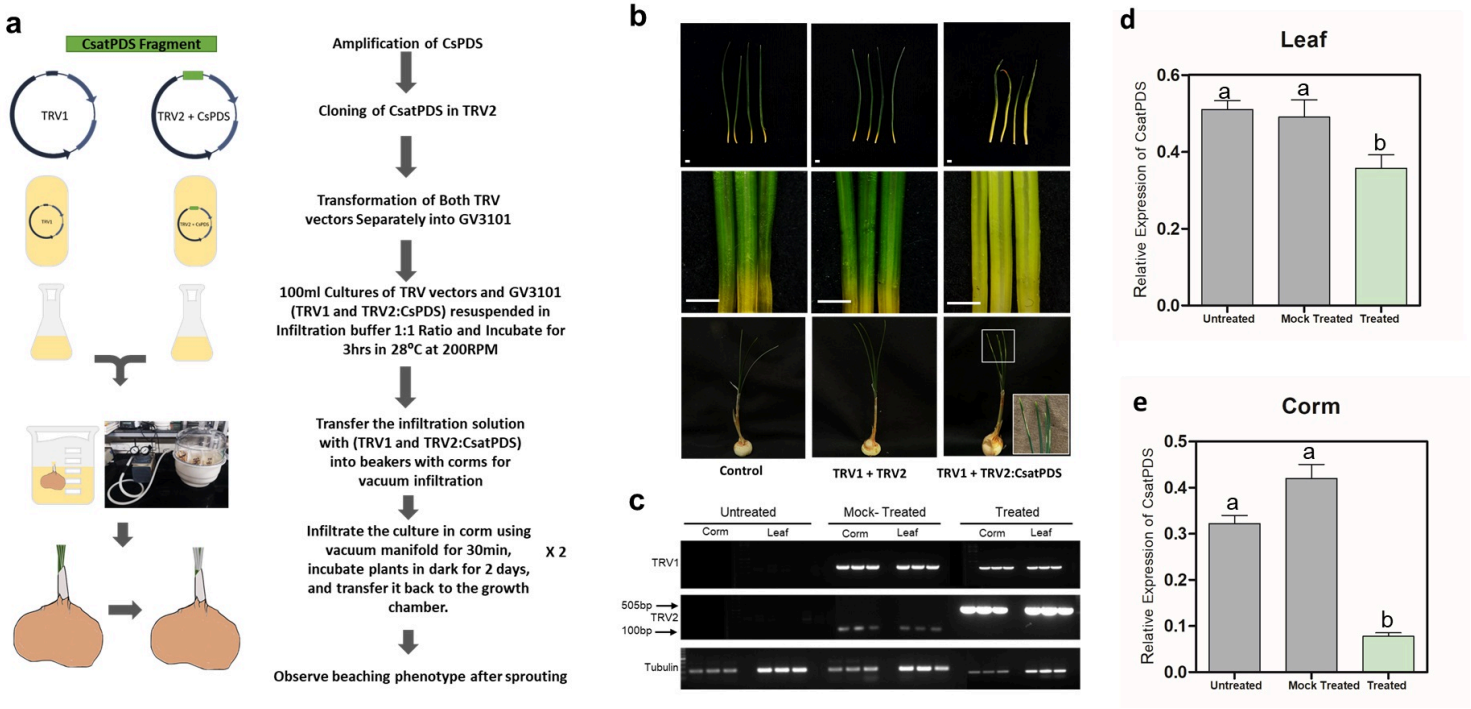


Figure 2

VIGS experiment targeting *CsatPDS* in *Crocus sativus* corms. (a) The schematic representation of workflow used for the study. It involves the construction of a vector system, the transformation of constructs into Agrobacterium strain GV3101, and the infiltration of culture in the dormant corms using vacuum infiltration. Bleaching phenotype was observed in newly developing leaves after sprouting. (b) Phenotypic observation of bleached leaves emerging after sprouting. The scale bar represents 3mm. The bleached phenotype was observed in young leaves as well as in later stages (c) Detection of TRV transcript by RT-PCR in VIGS photobleached leaves and inoculated corms of *Crocus sativus* after vacuum infiltration. TRV infection was present in both corm and leaf tissues (d) PDS silencing in corm and leaves tissues assayed by Quantitative Real-Time PCR. Expression values of the *CsatPDS* gene are averages for three biological replicates ± SEM, relative to the reference gene tubulin. Different letters a–b over the bars indicate significant differences at P < 0.005.