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EFFECTS OF SILENCING CYC2-LIKE GENES ON FLORAL DEVELOPMENT IN SOLANUM LYCOPERSICUM L. AND NICOTIANA OBTUSIFOLIA M. MARTENS & GALEOTTI (SOLANACEAE)

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

CYC- Cycloidea

DICH- Dichtoma

CYC2- Cycloidea 2

VIGS- Virus-induced gene silencing

TRV- tobacco rattle virus

WT- wild type

ECE- CYC2A

NEC- CYC2B

MCS- multiple cloning site

Bp- base pair

qPCR- Quantitative RT-PCR

SL – *So. lycopersicum*

NOB- *N.obtusifolia*

Abstract

EFFECTS OF SILENCING *CYC2-LIKE* GENES ON FLORAL DEVELOPMENT IN *SOLANUM LYCOPERSICUM* L. AND *NICOTIANA OBTUSIFOLIA* M. MARTENS & GALEOTTI (SOLANACEAE)

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

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CYCLOIDEA (*CYC)* and *DICHOTOMA* (*DICH*) of the *CYC2* clade of the TCP gene family have been shown to play a significant role in regulating the identity of the dorsal petals and abortion of the single dorsal stamen in *Antirrhinum majus*. It is believed that *CYC2*-like

genes are responsible for the convergent evolution of floral zygomorphy, but their role in the development of actinomorphic flowers is still unknown. In Solanaceae, previous analysis has identified two paralogs of *CYC2*-like genes*, CYC2A and CYC2B*, resulting from a gene duplication that predates the origin the family. Virus-induced gene silencing (VIGS) is a technique to study the gene function by silencing specific target genes of interest, which is shown to be useful in diverse plant species. Here, we report on the role of *CYC2*-like genes during floral development in Solanaceae based on the results of VIGS using tobacco rattle virus (TRV)-based vector in *Solanum lycopersicum* having completely actinomorphic flowers and *Nicotiana obtusifolia* having slightly zygomorphic flowers. Our VIGS experiments in *So. lycopersicum* show that downregulation of both *CYC2A* and *CYC2B* leads to misshaped petals, the unequal growth of the petals, and most frequently increased number of petals, stamens and sepals, while the carpel and ovule morphology remain the same as the wild type. On the contrary, downregulation of *CYC2A* and *CYC2B* in *N. obtusifolia* results in reduced number of flower organs in sepals, stamens, and petals, however carpels remained the same. For both solanaceous species, silencing *CYC2A* and *CYC2B* changes the property of cytoplasm and retards the rate of pollen germination. Our findings suggest that the *CYC2*-like genes are likely involved in the floral development, mainly regulating the number of floral organs and pollen development in Solanaceae.

Introduction

Flowers are a morphological innovation that leads to the great success of species and provides a critical topic for understanding the evolution of angiosperms (Mitchell-Olds & Schmitt, 2006). There are two major types of flower symmetry in angiosperms: actinomorphy (radial symmetry, regular flowers) that comprises more than one plane of floral symmetry, such as tomato flowers, and zygomorphy (bilateral symmetry, irregular flower) that consists only one plane of floral symmetry, such as the orchid. Floral zygomorphy is a highly homoplastic trait (Citerne, Jabbour, Nadot, & Damerval, 2010), which has evolved numerous times from actinomorphic-flowered ancestors during angiosperm evolution (Cubas, 2004) (Luo, Carpenter, Vincent, Copsey, & Coen, 1996). It is also thought to be an adaptive trait frequently associated with the clades with large species diversity (Cubas, 2004). With the advances in developmental genetics, we now have a better understanding of the genetic control of the development of floral zygomorphy, but we still know little about the function of this genetic pathway in actinomorphic flowers that is mainly due to no phenotypic changes observed in the *Arabidopsis* plants (Cubas, 2004).

Developmental genetic studies on *Antirrhinum majus* L. demonstrated that *CYCLOIDEA (CYC) and DICHOTOMA (DICH)* play a crucial role in the development of the dorsal petal identity and the abortion of the single dorsal stamen (Luo et al., 1996). Flowers of *A. majus* typically have five of petals, sepals, and four stamens (Luo et al., 1999). Floral zygomorphy,

however, is reduced in the *cyc* and *dich* single mutants and completely lost in *cyc*/*dich* double mutants. In double mutants, a slower rate of development was not observed in the dorsal region of floral development (Reeves & Olmstead, 2003). Furthermore, the dorsal and lateral petals resembled the ventral petals while the dorsal stamens become functional, and sometimes the flowers obtain additional petal and stamen (Gaudin, 2000, Hileman, 2014, Luo, 1999). Expression domain of *CYC* and *DICH* overlaps, and these genes show redundant function in floral development. *CYC* is expressed broadly in the two dorsal petals during early stage and thought to affect the growth rate and primordium initiation (Cubas, Coen, & Zapater, 2001). In contrast, *DICH* is narrowly expressed in the dorsal half of the two dorsal petals during early establishment of bilateral symmetry, but with less effect (Reeves & Olmstead, 2003). The function of *CYC*/*DICH* gene in specifying dorsal flower identity seems through regulating both cell proliferation and expansion (Luo et al. 1999; Gaudin et al. 2000).

CYC/*DICH* belong to the *CYCLOIDEA2* (*CYC2*), members of the TCP gene family of transcription factors, specifically in the lineage of the *CYC*/*TEOSINTE BRANCHED-1(TB1)* clade in the core eudicots and influence cell growth and proliferation (Howarth & Donoghue, 2006). TCP domain contains highly conserved regions and forms non-canonical basic helix-loop-helix (bHLH) structure (Reeves & Olmstead, 2003), which plays a role in nuclear localization of proteins, DNA binding, protein-protein interaction (Ma et al., 2014). Recent comparative expression and functional studies have demonstrated that *CYC2*-like genes were recruited independently many times in phylogenetic diverse core eudicots to evolve floral zygomorphy from actinomorphic-flowered ancestors, such as in Malpighiaceae (Zhang, Steinmann, Nikolov, Kramer, & Davis, 2013), Lamiales (Zhong & Kellogg, 2015), Fabaceae

(Fukuda, Yokoyama, & Maki, 2003), Asteridae (Reeves & Olmstead, 2003) and Asteraceae (Juntheikki-Palovaara et al., 2014). It has also been proposed that reversals to floral actinomorphy from floral zygomorphy are likely through modification or loss of *CYC2* gene expression through the regulatory evolution of *CYC2* or either the functional or regulatory changes in *CYC2*'s upstream regulators (Hileman 2014, Zhang et al. 2013). For two decades, *CYCLOIDEA2 (CYC2)*-like genes have been shown to play a vital role in the evolution and development of floral zygomorphy in core eudicots (Luo et al. 1996, Hileman 2014).

VIGS is a widely used approach for studying the reverse genetics. Certain procedures of VIGS are similar to transformation. Without tissue culture, however, VIGS is an easier and useful technique to study the gene function. There are many vectors currently available for VIGS (Senthil-Kumar, 2014). However, some vectors such as potato virus-x (PVX) based VIGS vector showed localized cell death and chlorosis, while tobacco rattle virus (TRV) based VIGS vectors showed fewer disease symptoms (Senthil-Kumar & Mysore, 2014). TRV has worked in various Solanaceous plants such as *N. benthamiana* (Ratcliff, Martin-Hernandez, & Baulcombe, 2001) and *So. lycopersicum* (Liu, Schiff, & Dinesh-Kumar, 2002). There are, however, limitations of TRV vector usages and the efficiency can be different depending on the plant species (Senthil-Kumar & Mysore, 2014). For successful infiltration, two virus vectors, TRV1 and TRV2, are required. TRV1 contains components required for viral movement and TRV2 hosts the target gene to be silenced. The TRV2 vector contains multiple cloning sites (Hubbard, McSteen, Doebley, & Hake), which allows inserting the desired length of genes. Both vectors are cloned into Agrobacterium, such as GV3101, as a carrier to infect the plants. Different sizes of DNA can be inserted, however, smaller than 23 bp DNA showed reduced efficiency (Burch-Smith,

Anderson, Martin, & Dinesh-Kumar, 2004). After transformation of the young seedlings, the phenotypes can be seen within 3-4 weeks and can affect early stage of plant development. However, VIGS cannot completely silence the gene expression, and phenotypical changes can be different between plants, which require multiple samples (Senthil-Kumar & Mysore, 2014). There are various methods can be applied for infiltrating plants using VIGS such as vacuum infiltration and injection. Depends on leaf types, different pressures of infiltration and various applications can be used. VIGS can be implemented when sprouts have emerged or when true leaves appear. However, the efficiency of silencing gene can be reduced if infiltration occurred during later stages of plant development.

Solanaceae (nightshades family) consist of ~3000 species in 99 genera, among which have many economically important plants, such as tomato, tobacco, potato, pepper, and eggplants (Knapp, 2010). Solanaceae provide us a great tool to illustrate the evolution of floral symmetry by studying the developmental and genetic basis of the trait, and understand the role of *CYC2* genes plays in floral development. One of the many advantages of studying the Solanaceae is that many species in the family have extensive genome information [e.g., DFCI (tomato, eggplant, tobacco, pepper, petunia, potato), Sol genomics network (Sato et al.), ESTobacco (tobacco)] and well-established tools. Also, species in this group consist of both actinomorphic and zygomorphic flowers (Knapp 2010). In particular, the RNAi-based virus-induced silencing (VIGS) approach works in many members of the family (Chung et al. 2004,Brigneti et al. 2004, Liu et al. 2002, Gossele et al. 2002)

Phylogenetic analysis of *CYC/TB1* clade in the Solanaceae indicates that each of *CYC1*, *CYC2*, and *CYC3* subclades have two gene copies. A possible genome-wide gene duplication

event predating the diversification of the family may result in these paralogs (Consortium et al. 2012, Bolger et al. 2014, Qin et al. 2014, Aversano et al. 2015, Zhang and Zhang 2015). For the *CYC2* clade, analysis of the sequence structure indicates that there are two sequence types in Solanaceae, *CYC2A (NEC)* and *CYC2B (ECE)*. *CYC2A* genes exhibit a unique *NEC* domain but missing or having a highly modified *ECE* domain. The recent work in Zhang lab on RNA *in situ* hybridization indicates that both *CYC2* paralogs are expressed in all five stamens in *So. lycopersicum* (Sato et al., 2012), which is opposite to its role in stamen abortion in *A. majus* (Zhang and Zhang. 2015). *So. lycopersicum* and other species in Solanaceae, therefore, provide us an excellent opportunity to dissect the role of *CYC2* in the development of actinomorphic flowers, which will shine lights on the origin and evolution of floral zygomorphy in angiosperms.

Here, we have sought to investigate the role of the two *CYC2* paralogs in the floral development of *So. lycopersicum* and *N. obtusifolia* using the VIGS approach. While the flowers of *So. lycopersicum* are completely actinomorphic, *N. obtusifolia* has slightly zygomorphic flowers. We examined the floral development in the plants with *CYC2* genes downregulated to identify the role that *CYC2* genes play. We found that the downregulation of *CYC2A* and *CYC2B* leads to the increased numbers of sepals, petals and stamens, and the retarded growth of petals in *So. lycopersicum*. In contrast, we observed the reduced numbers of sepals, petals and stamens, and modification of the ventral stamen in *N. obtusifolia*. In both species, the down regulation of *CYC2* genes leads to reduced rates of pollen germination. Together, our findings suggest that *CYC2*-like genes are likely involved in the floral development, especially influencing the number of floral organs and pollen development in Solanaceae.

Materials and Methods

Growing plants

Plastic pots and water trays were washed and bleached overnight before seeding. *So. lycopersicum* and *N. obtusifolia* seeds were planted in plastic pots with autoclaved Sungro Horticulture Professional growing B3 mix soils (Sungro, Agawam, MA). Until true leaves emerged, the tray was covered with plastic saran wrap plus plastic cover. Plants were watered by adding water to the tray, every three days, and were grown under 16-hour light cycles at room temperature.

Design and preparation of TRV2 vectors

Selected DNA regions of *CYC2* paralogs were cloned from the wild type of *N. obtusifolia* and *So. lycopersicum,* followed by Sanger sequencing to confirm the sequence identity (Figure S1). Targeted gene fragments were designed to produce specific siRNAs to of the targeted gene and to avoid off target gene for silencing. Phytoene desaturase (*PDS*) was designed from *N. obtusifolia* as positive control, which had insert size of 311 bp. *PDS* fragment was amplified using No_*PDS*_147F_XbaI and No_*PDS*_458R_SmaI. In *So. lycopersicum,* the fragments of 151 bps and 112 bps were used for *CYC2A* and *CYC2B* genes, respectively. A region with 35.3% DNA sequence similarity between *CYC2A* and *CYC2B* was used to silence these genes (Figure S1). For constructing the vector for *So lycopersicum CYC2A;CYC2B* double silencing, the two fragments from *CYC2A* and *CYC2B* were first connected with EcoRI (Table 1).

TRV2 vector was linearized with XbaII and SmaI (Table 1) within the multiple cloning sites. Inserting fragment with XbaII and SmaI sticky ends were cloned into the TRV2 vector. Gene specific primers *So. lycopersicum CYC2A* and *CYC2B* were used to confirm the successful insertion of the linked *CYC2A* and *CYC2B* fragments (Table 1). In *N. obtusifolia,* the fragments of 186 bp and 192 bp were used for *CYC2A* and *CYC2B*, respectively. A region with 40.2% DNA sequence similarity between *CYC2A* and *CYC2B* was used to silence these genes. The same methods were used to connect the *CYC2A* and *CYC2B* fragments and insert the combined fragment into the TRV2 vector. The TRV2 vector possessing the expected *N. obtusifolia CYC2A* and *CYC2B* insert was verified using PCR reaction (95°C for 10 min, 40 cycles of 95°C for 45 secs, 56°C for 45 secs, and 72°C for 1 min) using 156F (5'-TTACTCAAGGAAGCACGATGAGC-3') and 156R (5'-GAACCGTAGTTTAATGTCTTCGGG-3') primers to see if the construct is present within the multiple cloning sites of TRV2. Gel electrophoresis was used to analyze the PCR products, and the fragments were filter purified for sequencing to confirm the accuracy of the inserts.

VIGS on So. lycopersicum

For *So. lycopersicum*, while examining the *CYC2A;CYC2B* double gene silencing, we also carried out the *PDS* silencing as a positive control to determine the efficiency of transformation and the empty TRV2 vector as a negative control to determine phenotypic changes that may be induced by TRV. Inserted fragment of *PDS* was verified using *PDS* specific primers and empty vector was verified using 156F and 156R primers (Table 2). For each VIGS experiment, two vectors, i.e., TRV2-targeted-gene and TRV1, were transformed into the GV3101 strain of Agrobacterium, separately, using Bio-Rad Gene Pulser II Electroporation

System (Bio-Rad, Hercules, California). The transformed agrobacteria were then spread onto separate agar plates containing selective antibiotics, i.e., kanamycin (50 mg/mL in ddH_2O), gentamycin (50 mg/ mL in ddH_2O), and rifampicin (25 mg/mL in DMSO). The plates were incubated at 27°C for two days to allow the growth of single colonies. PCR was performed on the single colony to ensure that the cells on the plates were transformed with the expected TRV vectors. PCR primers to test TRV1 vector were OYL_195 (5′-CTT GAA GAA GAA GAC TTT CGA AGT CTC -3′) and OYL_198 (5′- GTA AAA TCA TTG ATA ACA ACA CAG ACA AAC -3′). Gene-specific PCR primers, SL_CYC2A_F (5'-CCTTGAGGAGCTCACTAATTGG -3') and SL_CYC2B_R (5'-GCAAAGGCTAGAGCAAGAGC-3') were to test TRV2 vectors. The presence of the fragments with expected size was tested using gel electrophoresis to determine the successful transformation.

Once the successful transformation was confirmed, the same single colony was picked and placed in an autoclaved culture tube containing 5 ml of Luria broth (LB) containing 5µL of each of the aforementioned antibiotics. The 5 ml cultures of agrobacteria with TRV1 or with TRV2-target-gene were shaken and incubated for one to two days at 27°C, and then were poured into separate flasks containing 500 ml of LB containing 500 µL of each of the three antibiotics. Flasks were gently shaken and incubated at 27°C until the optical density (OD) at 600 nm had reached 0.6-0.8 measured by Spectronic 20+ (ThermoFisher Scientific, Waltham MA). When the desired OD was achieved, each culture was spun down with centrifugation at 5000 revolutions per minute (rpm) for 10 min. Supernatants were discarded and pellets were re-suspended into 50 ml agroinfiltration medium containing 200 mM acetosyringone, 10 mM MES and 10 mM $MgCl_2$ and adjusted to pH 5.6. The re-suspended solution was placed in a sterile

conical tube, and gently shaken at room temperature for overnight. Then, OD of incubated mixture was measured using the Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, USA). When OD reached above 2.0, agroinfiltration media of agrobacteria with TRV1 and with TRV2-targeted-gene were mixed in a container with a 1:1 ratio of the volume. A solution of Vac-in stuff (Silwet-77 Cat. No. VIS-01c; Fisher Scientific, Hampton, NH) with a 0.05% (v/v) concentration was added into the container and mixed well, which is the final solution for plant infiltration.

The infiltration was performed on the seedlings roughly three weeks old with 3-4 true leaves. Plants were pulled out from the soil gently, loose soils were carefully shaken off, and leaves were placed in a plastic container submerged in the infiltration solution while roots were left out. Welch Duoseal 1402 Vacuum pump (Welch Vacuum Technology INC, Niles IL) was used to perform infiltration. Plants were vacuumed at -0.97 bars for two minutes three times. Afterward, plants were replanted into the soil and covered with plastic saran wrap to allow recovery. The phenotypes were evaluated when leaves change color in PDS and when both the VIGS mutants and the wild types (WT) begins to flower.

VIGS on N. obtusifolia

For *N. obtusifolia,* the *PDS* and *CYC2A;CYC2B* silencing plants were prepared similarly as the preparation described for *So. lycopersicum*. For TRV2, PCR primers, NOB_CYC2A_F (5'-CCTAGCAAAACCCTCGATTGG-3') and NOB_CYC2B_R (5'-TTTGATTGCGAGAGAGTCCA-3'), were used to verify the presence of the fragments with expected size of *CYC2A;CYC2B* cloned into agrobacteria. We had modified the protocol of infiltration since *N. obtusifolia* had softer leaves compared to *So. lycopersicum* and Welch

vacuum pump was too powerful that killed the plants. Therefore, we used the Gast vacuum pump (Gast Manufacturing, INC, Benton Harbor, MI) at -0.78 bars for two minutes three times for *N. obtusifolia*.

VIGS on Schizanthus grahamii

The *PDS* was prepared with the same method. The plants of *S. grahamii* were infiltrated using the injection method instead. 1 ml of syringe tube was used. Infiltration medium was injected directly into the abaxial surface of the leaf. Infiltration was performed until the entire region of the leaf blade changed to darker green. Plants were recovered, and the phenotypic changes were evaluated in three weeks.

Tests of pollen viability

Two methods were used to evaluate the pollen viability. (1) Alexander's stain (Alexander, 1969) was prepared with 10 ml of 95% alcohol, 10 mg Malachite green, 25 ml Glycerol, 5 g phenol, 5 g Chloral hydrate, 50 mg acid fuchsin, 5 mg of Orange G, 1 ml Glacial Acetic acid, and 50 ml distilled water. The stain was stored at room temperature in a glass bottle wrapped in aluminum foil. Pollens were spread on glass microscope slides, and stains were placed on top to cover the pollens. After at least four hours incubation at room temperature, the cover slide was added, and pollens were observed using a light microscope. (2) A method by Karapanos et al. (2006) was used to examine pollen germination. The pollen germination medium was prepared in a total volume of 100 ml containing 1% (w/v) agar, 15% (w/v) sucrose, and 50 mg/L H_3BO_3 in water. All components other than the boric acid were dissolved in a flask using a microwave. Once everything was dissolved, the flask was placed on a hot plate, and boric acid was added

while stirring. Germination medium was poured evenly on glass slides placed in a petri dish. Once the medium solidified on the slides, pollens from the wild type and mutant flowers were dusted from their stamens onto the slides. The plates were covered and incubated overnight at room temperature, which is followed by staining with Alexander's stain for at least 1 hour. The cover slides were placed, and the slide was cut out of the plate using blades. Slides were carefully lifted from the plate, and the excess gel was cut around the slide. After the slides had been wiped using Kim wipes, slides were observed under Zeiss Axio Imager microscope (Zeiss United States of America, Columbus OH) with Zeiss Axiocam Icc5 to image pollen germination. Germination tube lengths were measured using Image J software (Schindelin et al., 2012).

Statistical analysis of pollen tube length

Statistical analysis on pollen germination were formed with R software (R-project.org) using linear mix model with ANOVA to test fixed effect using Type III with Satterthwaite approximation for degrees of freedom. Relative lengths of pollen tubes and standard errors of means were calculated.

Quantitative RT-PCR

The late stage flowers (~1 cm in length) of *N. obtusifolia* were collected by snap frozen in liquid nitrogen and stored in a - 80ºC freezer. RNAs were extracted from the flower buds using the RNAqueous® total RNA isolation kit (Invitrogen, Carlsbad, CA). DNA contamination was removed using DNAfree kit (Ambion, Foster City, CA). cDNA synthesis was performed using ImProm-II™ Kit (Promega, Madison, WI). qPCR was carried out with SYBR Taq using Bio-Rad CFX Connect Real-Time PCR (Bio-Rad, Hercules, CA). Beta-tubulin was

used as an internal control of the level of gene expression. The error bars resulted from nine data points generated from three independent flower samples of the same individual and three qPCR technical repeats for each sample.

Results

Phonotypic changes of CYC2A;CYC2B double silencing plants in So. lycopersicum

Plants infiltrated with 2-3 true leaves result in more noticeable phenotypic changes compared to plants infiltrated with 4-5 true leaves, which suggests that infiltration is more effective at early stages of plant development. Most of the plants were able to recover after the infiltration, but with young plants, they were more susceptible to die within a week. Phytoene desaturase (PDS) was successfully downregulated in *So. lycopersicum* resulting in a bleached phenotype in leaves (87.5% of 56 plants that survived the infiltration) (Figure 2). Plant infiltrated with empty vector showed no morphological changes compared to the wild type. Overall, the growth rate was slow in double silencing plants compared with the wild types, which leads to the double silencing plants flowered later than the wild-type plants. The plants with *PDS*-silenced, or the empty vector flower earlier than double *CYC2A;CYC2B* double silencing plants. When compared with wild type, the empty vector flowered the same time as wild type, while *PDS* flowered week later.

We found the increased number of floral organs, morphological changes in petals, and retardation of pollen germination (Figure 3). Twelve plants (26.6% of the 45 plants that survived the infiltration) show abnormality in floral morphology. The most common phenotypic change observed is the flowers with six parts in petals, sepals and stamens (Figure 3B and E). The morphological changes were also observed in petals. We observed flowers with the black mark

on midvein of each petal (Figure 3B) or with the abortion of one or two petals (Figure 3C and D). The petals of these flowers are also strongly curved, and the sepals shifted to display in a non-actinomorphic way. For the flowers with aborted petal(s), there is no change in numbers in sepals or stamens observed.

We tested if silencing *CYC2s* would cause stamens to be aborted since *CYCs* was expressed in all five functional stamens. However, there was no stamen abortion observed. We, therefore, examined if the development of pollen was altered through comparing the pollens between the wild type (7 plants), empty vector (5 plants), and double silencing plants (10 plants) (Figure 4). We found significant changes of pollen contents and the rate of pollen germination (Kim et al. 2016). For the pollen staining, the pollen coat always shows a bluish-green color in wild-type, empty vector, and double silencing plants. The cytoplasm of pollens from the wild type show light purple staining, while VIGS *CYC2A;CYC2B* plants show dark purple staining in the cytoplasm during the first trial. However, during the second trial, empty vector and VIGS *CYC2A;CYC2B* cytoplasm showed similar stain as the wild type. Furthermore, the pollen germination tests show that pollen tubes of *CYC2A;CYC2B* were reduced by 28.9% ($F_{df1,df2}$ =4.892, P= 0.03622) compared to the wild type while the empty vector and the wild type show similar length in pollen tube length (Figure 6). These results suggest that the contents in cytoplasm might be changed in the *CYC2A;CYC2B* double silencing plants, which might influence pollen germination. The nonviable pollens that only have the empty pollen coat can be found in wild-type, empty vector and *CYC2A;CYC2B* silencing plants.

Phenotypic changes of CYC2A;CYC2B double silencing plants in N. obtusifolia

Phytoene desaturase (PDS) was successfully downregulated in *N. obtusifolia* resulting in a bleached phenotype in leaves (91.3% of 23 plants that survived the infiltration) (Figure 7).

For *CYC2A;CYC2B* silencing plants of *N. obtusifolia,* by contrast, we found the decreased number of floral organs, and the changes of size in ventral stamen (Figure 8). Nine plants (42.8% of the 21 plants that survived the infiltration) of *N. obtusifolia* show abnormality in floral morphology. The most common phenotypic changes observed (77% of the plants seen morphological changes) are the flowers that aborted one part in petals, sepals, and stamens, and the corolla became square-like (Figure 8D). Some of the flowers carried phenotypic changes that help imply which organ is aborted. For examples, we saw one flower with an enlarged single dorsal petal shifted to one lateral petal, which was slightly reduced in size (Figure 8B). For the same flower, the position of the lateral sepal close to this reduced lateral petal slightly shifts to the ventral position (Figure S2A). In another flower, the square-like corolla had the residue of a lateral petal (Figure S2 B). These results suggest one of the two lateral petals were reduced through silencing *CYC2* genes. Also, one of the two dorsal stamens seemed aborted (Figure 8B). Other than the reduction of stamens, we also observed the phenotypic changes in the short ventral stamen. In one plant, it shows that the ventral stamen is equal in length and size of the other stamens (Figure 8C).

Similarly, we examined if the development of pollen was altered through compared the pollens between the wild type and the double silencing *N. obtusifolia* plants (Figure 9). For the pollen staining, similar as *So. lycopersicum,* the wild type shows lighter purple in the cytoplasm and VIGS *CYC2A; CYC2B* shows dark purple. These results suggest that the contents in

cytoplasm might be different between these plants. Furthermore, the pollen germination tests show that the germination tube of VIGS *CYC2A;CYC2B* plants (Figure 10) are reduced by 34.3% (F_{df1} =15.963, P= $9.266x10^{-5}$) compared to the wild type (Figure 11). The nonviable pollens that only have the empty pollen coat can be found in both wild-type, and *CYC2A;CYC2B* silencing plants.

The qPCR was performed to see if there is a correlation between the level of *CYC2* expression and the degree of changes in phenotypes. We showed that the overall level of *CYC2* expression was reduced in the *CYC2A;CYC2B* silencing plants compared to the wide-type plants (Figure 12A). The level of gene expression is decreased by ~ 40% for *CYC2A*, and by ~ 75% for *CYC2B*. Plants with significant changes of the floral morphology show much-reduced expression level of *CYC2A* and *CYC2B* compared to the plant with weak phenotypic changes (Figure 12B and C).

Successful PDS silencing in S. grahamii

The *PDS* was successfully downregulated in *S. grahamii* (Figure 13). The same *PDS* designed for *N. obtusifolia* was used. Leaves show the white bleach phenotype, which indicates the protocol with injection infiltration modified for *S. grahamii* is sufficient to downregulate the gene of interest. We, therefore, will use this method to study the function of *CYC2* genes in this Solanaceous species with strongly zygomorphic flowers.

Discussion

The role of *CYC2* genes in floral development - The role of floral symmetry genes on the development of actinomorphic flowers is still unknown. The *CYC/DICH* are members of the TCP gene family of transcription factors that play a role in transcription regulation (Luo et al., 1996). In *A. majus*, it suggests that *CYC/DICH* is expressed at early stage floral meristem until the late stages of floral development and their roles include the effects during primordium initiation and regards cell growth (Luo et al., 1996). The double mutants of *CYC/DICH* can also lead to increase the number of organs. Similarly, we have seen the increased number of floral organs in the downregulation of *CYC2* in *So. lycopersicum*. In contrast, the influence of the number of floral organs of *N. obtusifolia* is opposite when *CYC2* was silenced. Instead of observing the increased number of floral organs, the number of the floral organs is reduced by one in most phenotype observed. These results suggest that *CYC2* likely plays a role in regulating floral merosity.

Previous analyses of *CYC2* phylogeny in Solanaceae have indicated that there are two subclades of *CYC2,* i.e., *CYC2A* and *CYC2B* (Zhang and Zhang 2015). These *CYC2* paralogs result from a gene duplication that predates the origin of Solanaceae. The express of *CYC2* paralogs was detected during floral development in *Schizanthus pinnatus* Ruiz & Pav., *N. obtusifolia*, and *So. lycopersicum* (Zhang and Zhang 2014, Zhang and Zhang 2015). In *So. lycopersicum*, both *CYC2* paralogs are expressed in pollen mother cells of five functional

stamens, which is opposite to its role in stamen abortion in *A. majus*. RNA *in situ* hybridization studies show that one of the two *CYC2A* copies, which resulted from a *Schizanthus*-specific gene duplication, of *Sc. pinnatus* is expressed within pollen mother cells and ovules. Interestingly, the preliminary VIGS experiments indicate that downregulation of this *CYC2A* copy leads to the functional stamens bearing empty anthers and shortened filaments (Zhang and Zhang 2015).

Downregulation of *CYC2* genes delays the plant growth – We found that the *CYC2A;CYC2B* silencing plant grew slower than the wild type and flowering was delayed for three weeks. Previous studies showed that the TRV vector has little interference of growth. Furthermore, our empty vector infiltrated *So. lycopersicum* does not show delayed growth (Senthil-Kumar, 2014). Therefore, this observation suggests that *CYC2* genes may promote the plant growth in Solanaceae through positive regulation of cell growth.

Notes on VIGS methods - VIGS is an excellent method to study the function of the gene for reverse genetics. Depending on the type of plant and leaves, the VIGS approach is required to optimize the methods of infiltration. Since it does not rely on stable transformation, no tedious tissue cultures are needed, and the gene functions can be quickly evaluated (Meng et al., 2016). Even though it is easy to use, the different phenotypes usually are observed due to the variation of gene downregulation in different plants and different parts of the same plant. Therefore, the VIGS experiments typically require many replicates of samples, and repeats of trials (Figure 9). VIGS allows to silencing the specific region of the gene, but does not yield a complete loss of gene expression (Senthil-Kumar, 2014). It is important to choose the right size and fragment that is unique to the gene of interest that produce specific siRNA (Senthil-Kumar, 2014). We need to design the experiments and interoperate the data with care using the VIGS approach since the

possibility of the closely related paralogs might also be downregulated simultaneously. Unlike *PDS* where the bleached leaf phenotype is easy to be identified, the *CYC2A;CYC2B* double silencing does not show if the successful infiltration or determine the gene downregulation in certain locations. Therefore, *Anthocyanidin Synthase (ANS)* could be used to determine the successful downregulation of genes in flowers that will show the white bleached floral phenotype. However, this method cannot be used when the flower is white or lacking *ANS* expression. TRV vector with green fluorescent protein is available and can be helpful to figure out the region of the plants carrying these vectors. Previous studies showed that GFP inserted TRV2 vector serves better control than empty vector, which suggests using GFP inserted TRV2 vector could help to find the expression region in our future research (Senthil-Kumar, 2014).

Future directions - Our research of *CYC2* in both *So. lycopersicum* and *N. obtusifolia* starts to provide a better understanding on the role of *CYC2* in floral development. Future research of these species with single gene silencing using *CYC2A* or *CYC2B* will help further dissecting the role of these paralogs. The recent studies showed that *PDS* was successfully downregulated on *S. grahamii*, which suggest that this species can be used to study the role of *CYC2* genes in the zygomorphic-flowered species of Solanaceae.

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APPENDIX

Table 1. Primers designed with a restriction enzyme cutting site for preparing TRV2 vectors

	Primer Name	Primer Sequence
Positive Control	No_*PDS*_147F_XbaI	CTT CTA GAC CCC AAT ATG CAG AAC CTG T
	No_*PDS*_458R_SmaI	ATC CCG GGA CCC TAT CAG GCA CAC CTT G
N. obtusifolia	NOB_*CYC2B*_TRV2_F_EcoR1	TAG AAT TCG GCT ATT CAC AAA GTC CAA A
	NOB_*CYC2A*_TRV2_R_EcoR1	CTG AAT TCT CTT TCC GAA CCC TCA TTT G
	NOB_*CYC2B*_TRV2_F_XbaI	CTT CTA GAT GGC TAT TCA CAA AGT CCA AA
	NOB_*CYC2B*_TRV2_R_SmaI	ATC CCG GGT GGA CTC TCT CGC AAT CAA A
	NOB_*CYC2A*_TRV2_F_Xbal	CTT CTA GAC TAG CAA AAC CCT CGA TTG G
	NOB_*CYC2A*_TRV2_R_SmaI	ATC CCG GGT CTT TCC GAA CCC TCA TTT G
So. lycopersicum	SL_*CYC2B*_TRV2_F_EcoR1	TAG AAT TCT CAT CAA CGA TGT GTC AAA GAA
	SL_*CYC2A*_TRV2_R_EcoR1	CTG AAT TCT GTT ACT TCT TTT GCT CTT TTG G
	SL_C*YC2B*_TRV2_F_Xbal	CTT CTA GAT CAT CAA CGA TGT GTC AAA GAA
	SL_CYC2B_TRV2_R_SmaI	ATC CCG GGG CTC TTG CTC TAG CCT TTG C
	SL_*CYC2A*_TRV2_F_Xbal	CTT CTA GAC CTT GAG GAG CTC ACT AAT TGC
	SL_*CYC2A*_TRV2_R_SmaI	ATC CCG GGT GTT ACT TCT TTT GCT CTT TTG G

Table 2. PCR primer used to verify the fragment in agrobacteria

	Primer Name	Primer Sequence
TRV1	OYL195	CTT GAA GAA GAA GAC TTT CGA AGT CTC
	OYL 198	GTA AAA TCA TTG ATA ACA ACA CAG ACA AAC
TRV2	156F	TTA CTC AAG GAA GCA CGA TGA GC
	156R	GAA CCG TAG TTT AAT GTC TTC GGG
PDS	NOB_PDS_F	ACC CCA ATA TGC AGA ACC TGT
	NOB_PDS_R	CAA GGT GTG CCT GAT AGG GTG
So. lycopersicum	SL_CYC2A_F	CCT TGA GGA GCT CAC TAA TTG G
	SL_CYC2A_R	CCA AAA GAG CAA AAG AAG TAA CA
	SL_CYC2B_F	TCA TCA ACG ATG TGT CAA AGA A
	SL_CYC2B_R	GCA AAG GCT AGA GCA AGA GC
N. obtusifolia	NOB_CYC2A_F	CCT AGC AAA ACC CTC GAT TGG
	NOB_CYC2A_R	CAA ATG AGG GTT CGG AAA GA
	NOB_CYC2B_F	GGC TA TT CAC AAA GTC CAA A
	NOB_CYC2B_R	TTT GAT TGC GAG AGA GTC CA

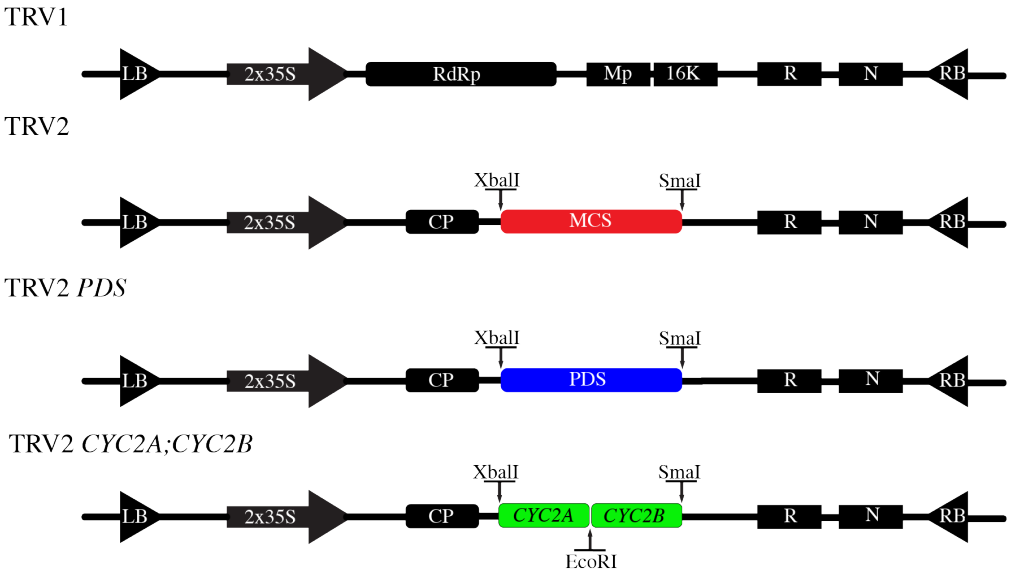


Figure 1. Schematic of VIGS TRV1 and TRV2 vector constructs. TRV2 vectors were designed accordingly. Left boarder (LB), RNA-dependent RNA polymerase (RdRp), Coat Protein (CP), Movement Protein (MP), 16Kd protein (16K), Self-cleaving Ribozyme (R), NOS terminator (N), Right Boarder (RB), Multiple Closing Site (Hubbard et al.), Phytoene desaturase (*PDS*)

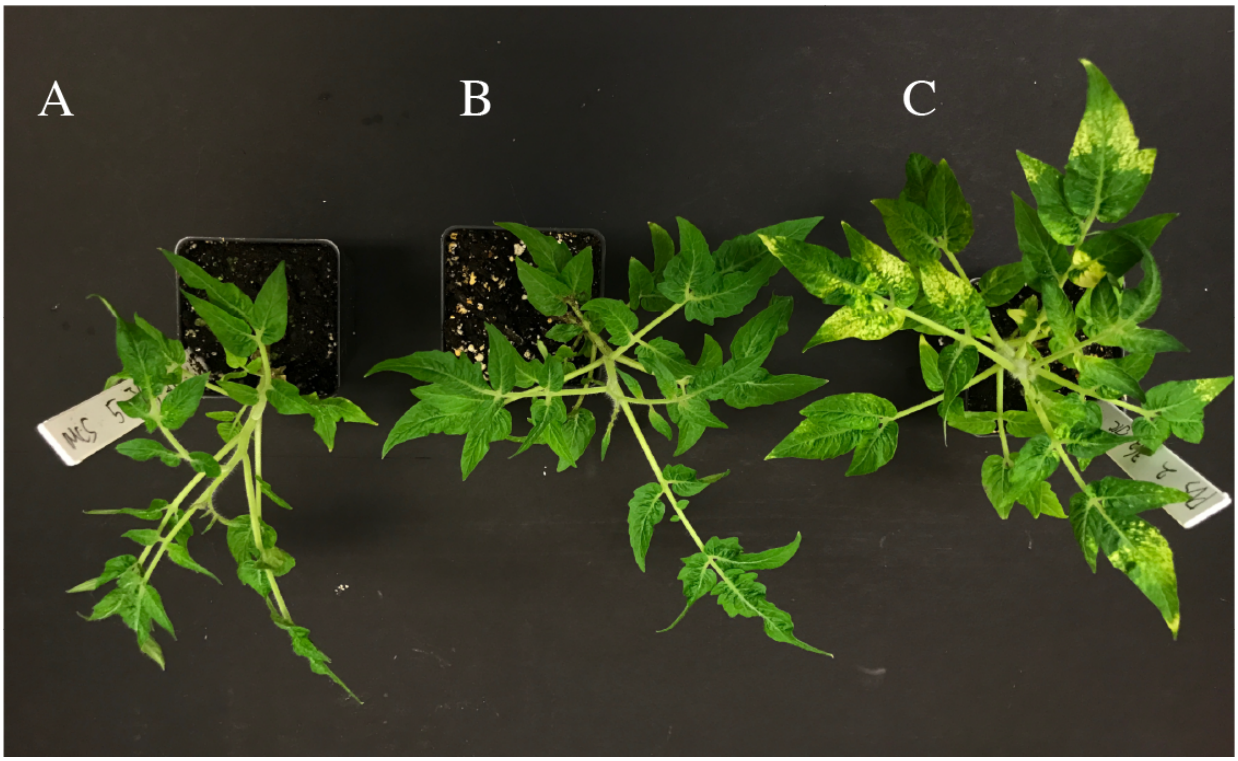


Figure 2. Positive and negative control of VIGS experiment of *So. lycopersicum*. MCS(A), Wild type (B), Phytoene desaturase (*PDS*) (C). *PDS* was successfully down regulated in *So. lycopersicum*. *PDS* (positive control) plants showed lighter green in the newly developed leaves two weeks after the infiltration. MCS (negative control) showed no phenotypic changes compare to wild type.

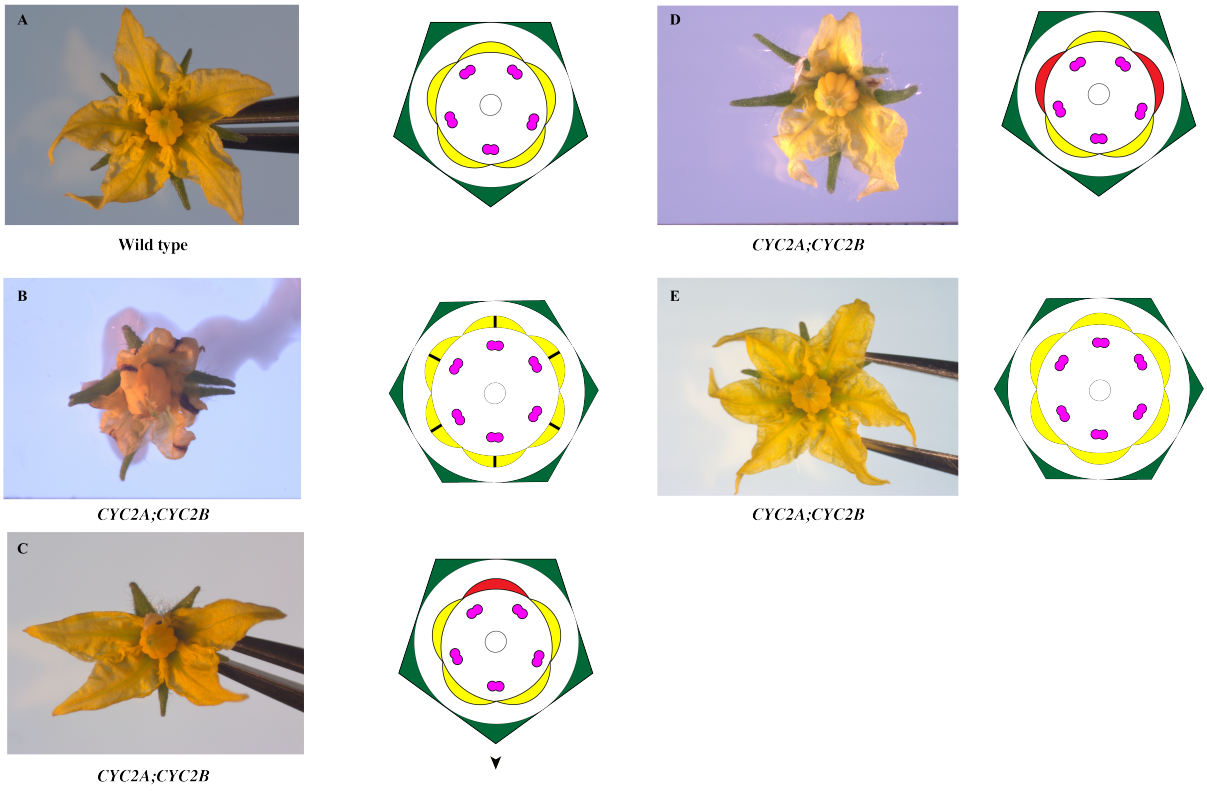


Figure 3. Phenotypes of flowers observed in the wild type (A) and VIGS *CYC2A;CYC2B* (B-E) of *So. lycopersicum*. *CYC2A;CYC2B* silenced *So. lycopersicum* flowers show changes in the number of petals, sepals and stamen. (A) Wild-type flower; (B) black mid-vein on petals (1 out of 45 plants) ; (C) one aborted petal (2 out of 45 plants); (D) two aborted petals (1 out of 45 plants) ; (E) six petals, stamens, sepals (8 out of 45 plants). Floral diagrams are illustrated right next to flowers. Aborted petals were colored in red.

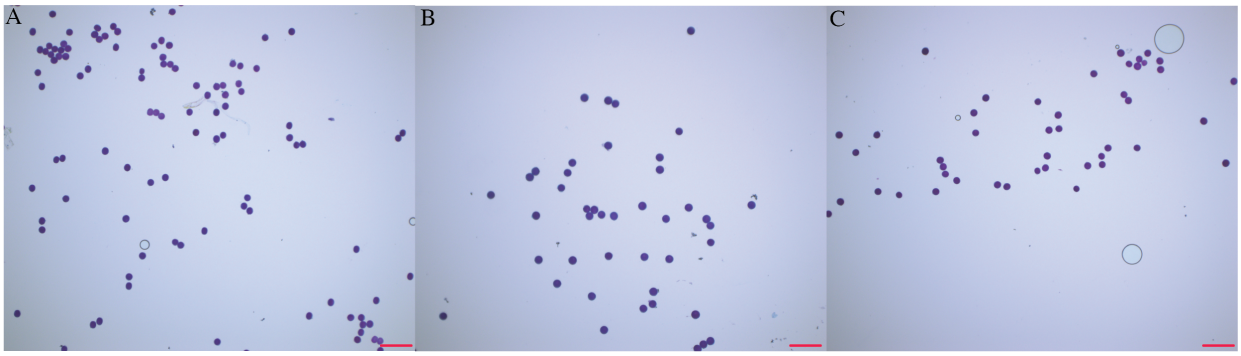


Figure 4. Pollens of wild type (A), empty vector (B) and VIGS *CYC2A;CYC2B* (C) of *So. lycopersicum*. Pollens from wild type (A), empty vector (B) and VIGS *CYC2A;CYC2B* (C) were stained using Alexander's staining for at least 4 hours. Scale bars = 0.1 mm.

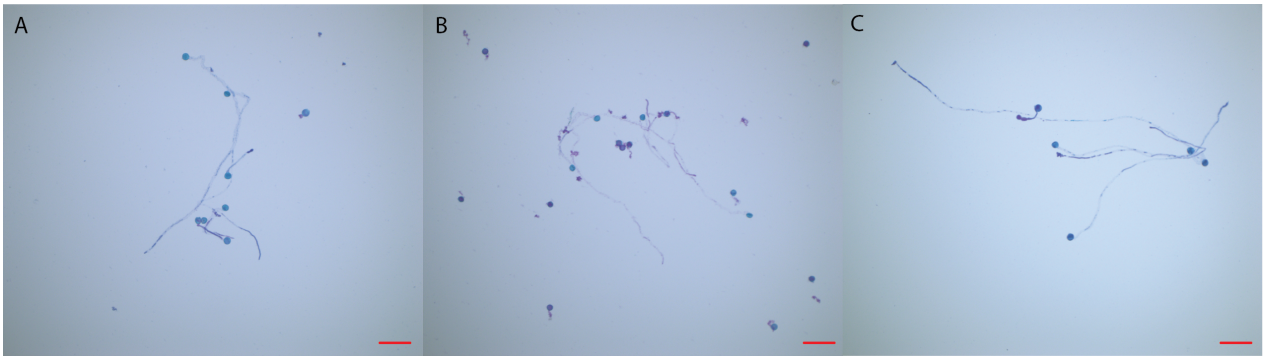


Figure 5. Pollen germination of wild type (A), empty vector (B) and VIGS *CYC2A;CYC2B* (C) of *So. lycopersicum*. Pollen germination of the wild type, empty vector, and VIGS *CYC2A;CYC2B* of *So. lycopersicum* was tested after 12-hour incubation of pollens in the pollen germination medium. Results were observed after staining with Alexander's stain. Scale bar = 0.1mm

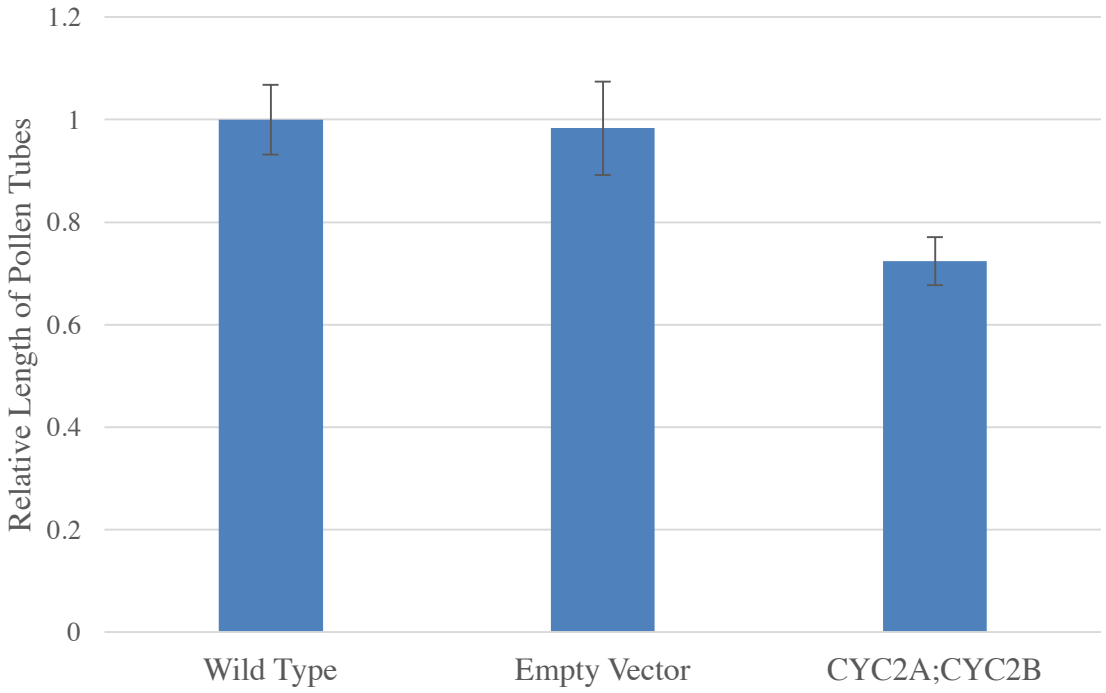


Figure 6. Statistical analysis of pollen tube length in wild type and VIGS *CYC2A;CYC2B* of *So. lycopersicum*. The length of pollen tubes was measured using Image J software (Schindelin et al., 2012).

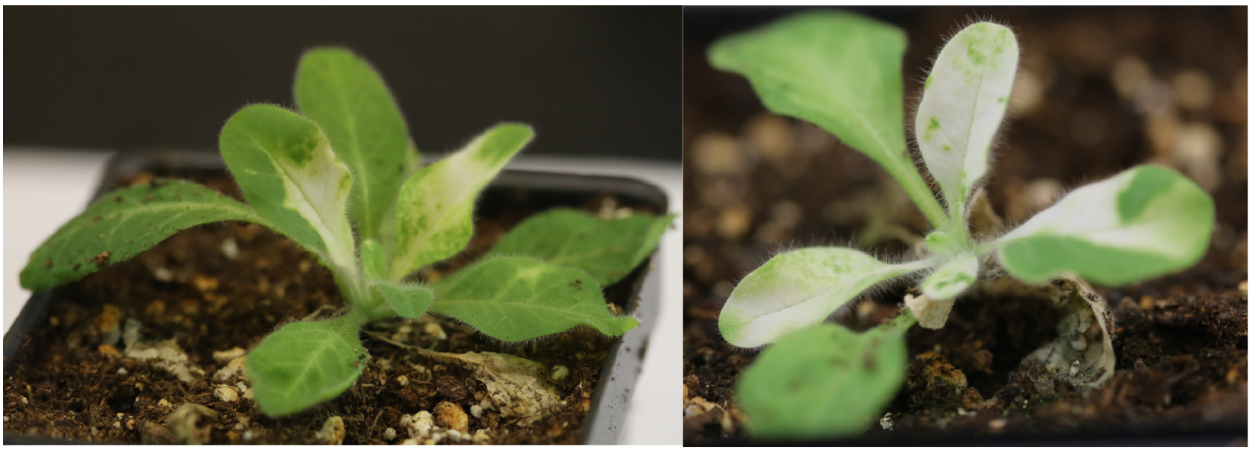


Figure 7. Positive control of VIGS experiment of *N. obtusifolia*. *PDS* was successfully downregulated in *N. obtusifolia*. 91% of infiltrated plant showed bleached leave

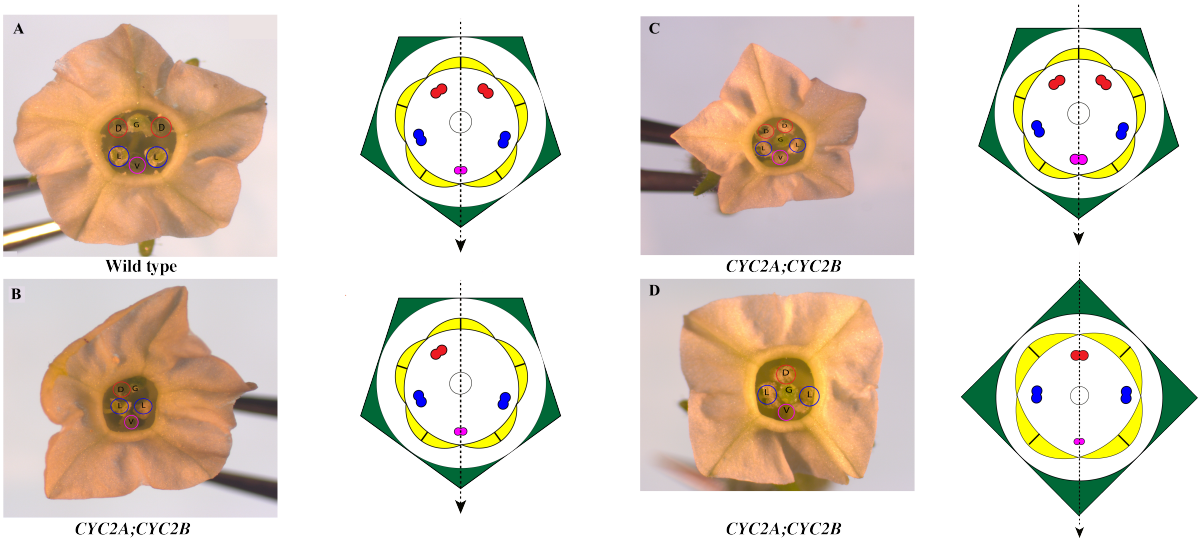


Figure 8. Phenotypes of flowers observed in the wild type (A) and VIGS *CYC2A;CYC2B* (B-D) of *N. obstuillia*. *CYC2A;CYC2B* silenced *N. obstuillia* flowers show changes in the number of petals and stamens. (A) Wild-type flower has five petals, four long dorsal and lateral stamens, and one short ventral stamen. (B) a flower shows one stamen aborted in dorsal region and maintains five petals (1 out of 21 plants). (C) a flower shows all stamens similar in length (1 out of 21 plants), (D) a flower shows four petals instead of five petals and reduced one stamen in dorsal region (7 out of 21 plants). Floral diagrams are illustrated right next to flowers. Different sections of stamens are color coded. D, dorsal in red; L, lateral in blue; V, ventral in pink; G, gynoecium

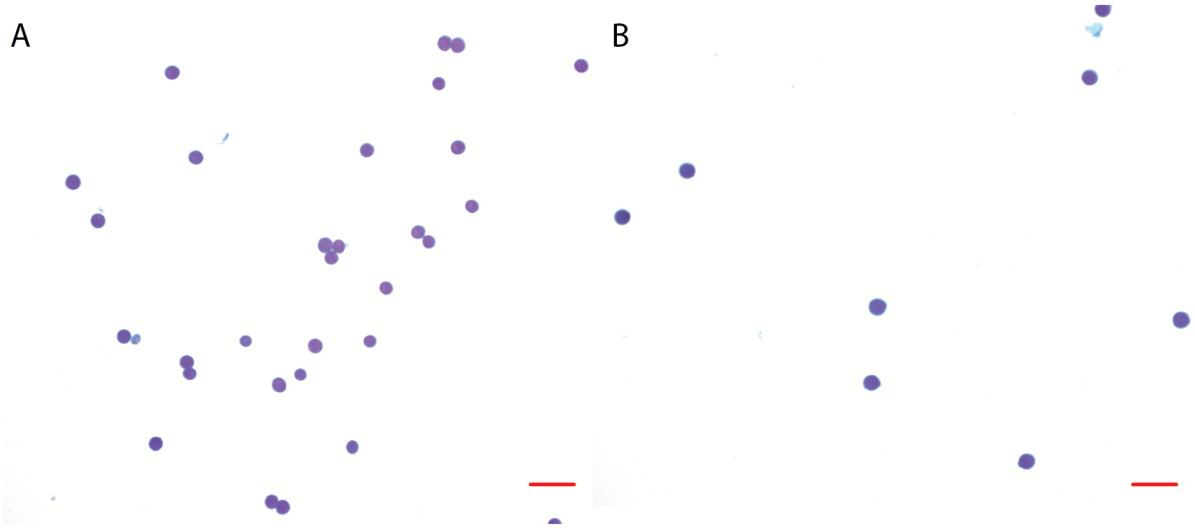


Figure 9. Pollens of wild type (A) and VIGS *CYC2A;CYC2B* (B) of *N. obtusifolia*. Pollens from wild type (A) and VIGS *CYC2A;CYC2B* (B) were stained using Alexander's staining for at least four hours before imaging. Scale bars = 0.1 mm.

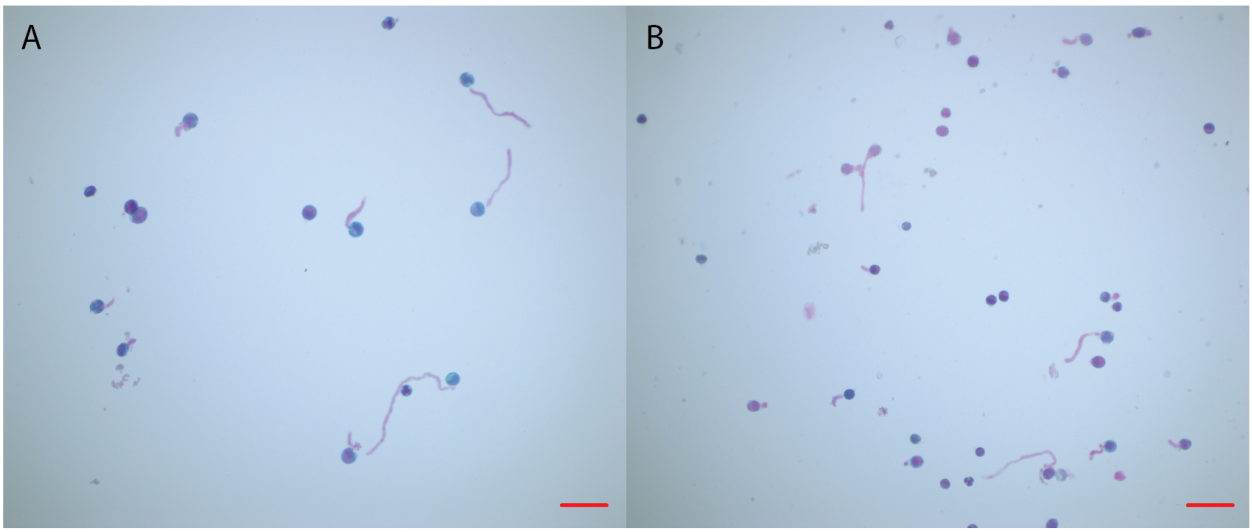


Figure 10. Pollen Germination of wild type (A) and VIGS *CYC2A;CYC2B* (B) of *N. obtusifolia*. Pollen germination of the wild-type and VIGS *CYC2A;CYC2B* plants of *N. obtusifolia* was tested after 12-hour incubation in the pollen germination medium. Results were observed after staining with Alexander's stain. Scale bar = 0.1mm

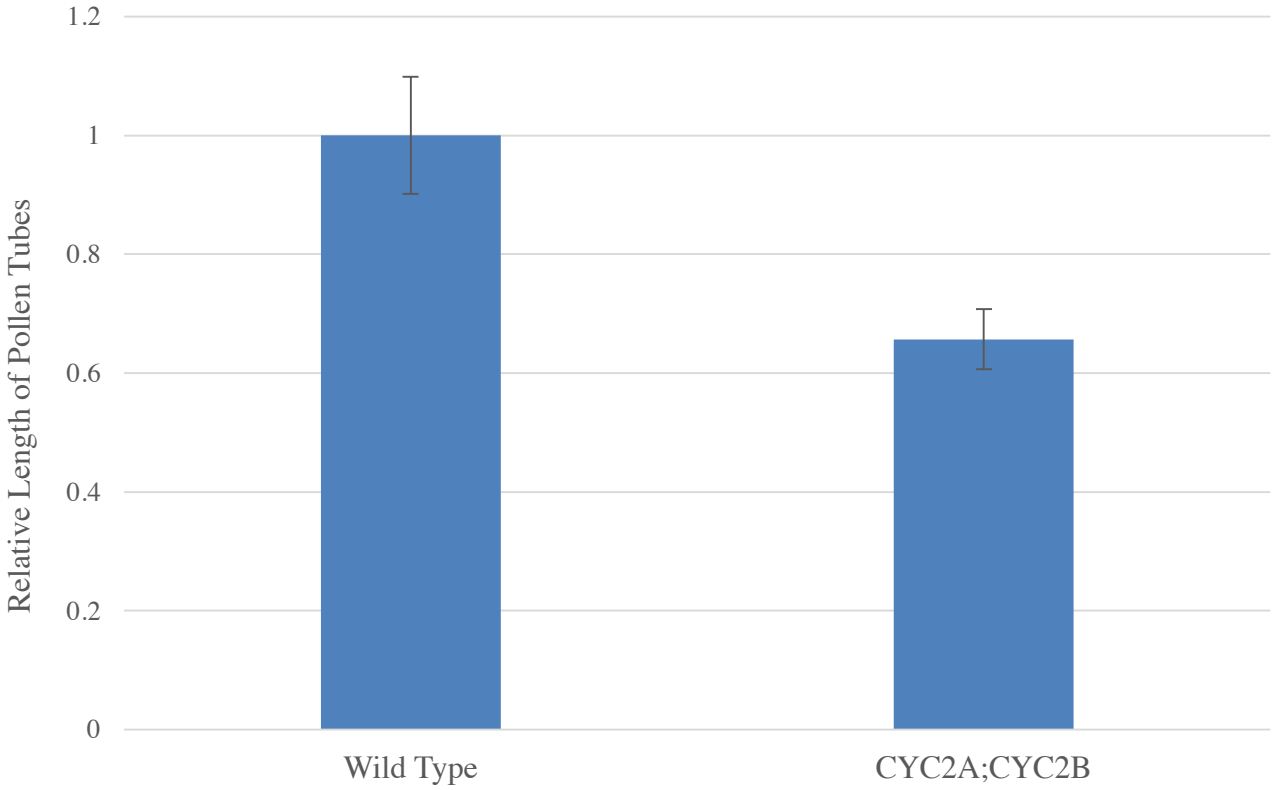


Figure 11. Statistical analysis of pollen tube length in wild type and VIGS *CYC2A;CYC2B* of *N. obtusifolia*. The length of pollen tubes was measured using Image J software (Schindelin et al., 2012).

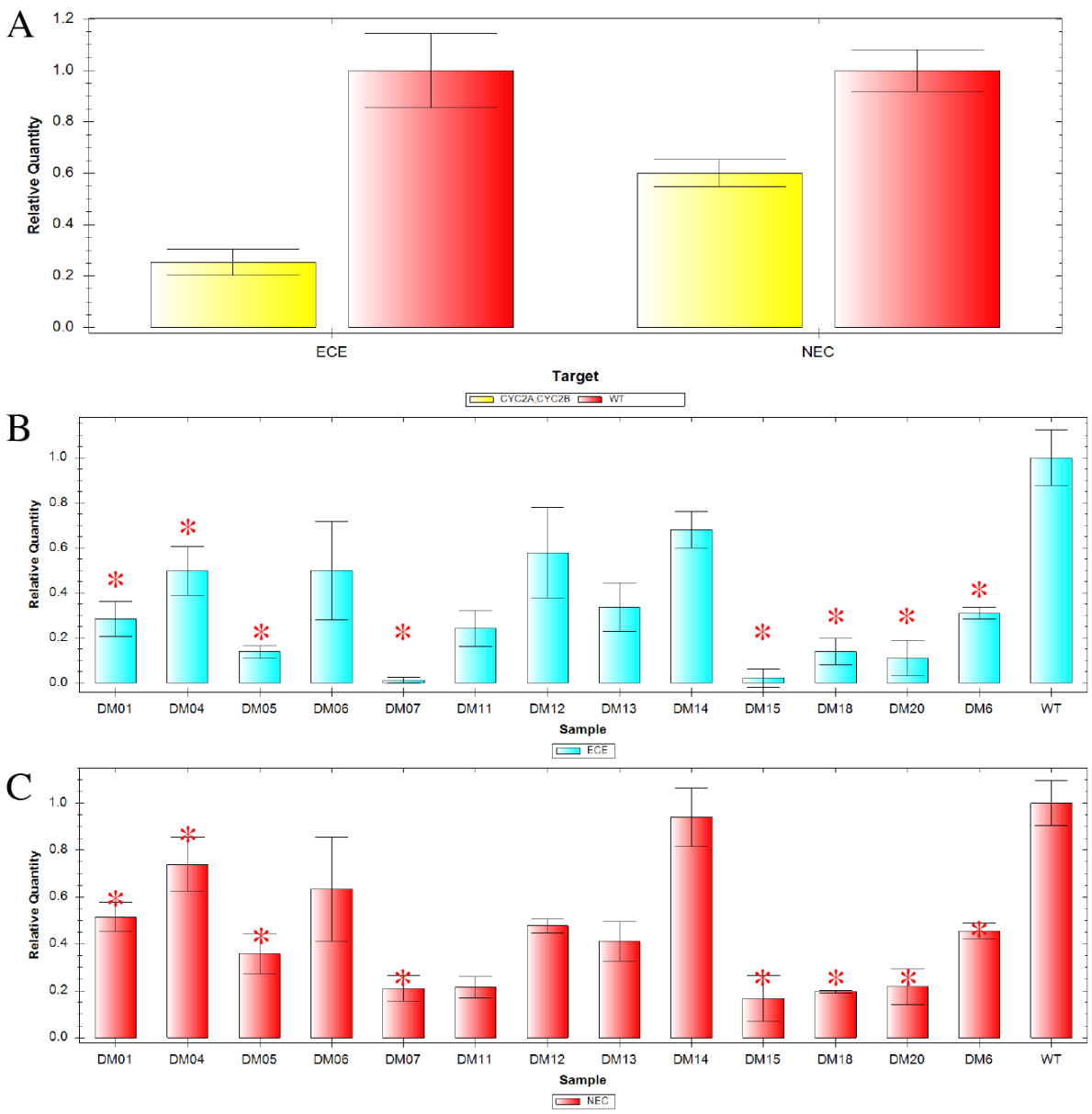


Figure 12. Relative expression level of *CYC2A(NEC)* and *CYC2B(ECE)* genes in the double mutant and wild type plants. RNA was extracted from both the double mutant and wild type (bud length ~1cm) and performed RT-qPCR. Tubulin was used as a control. Over all, CYC2A expression level was decreased by 40% and *CYC2B* expression level was decreased by 75%. VIGS plants with altered phenotypes showed significant low expression of both *CYC2A* and *CYC2B*.

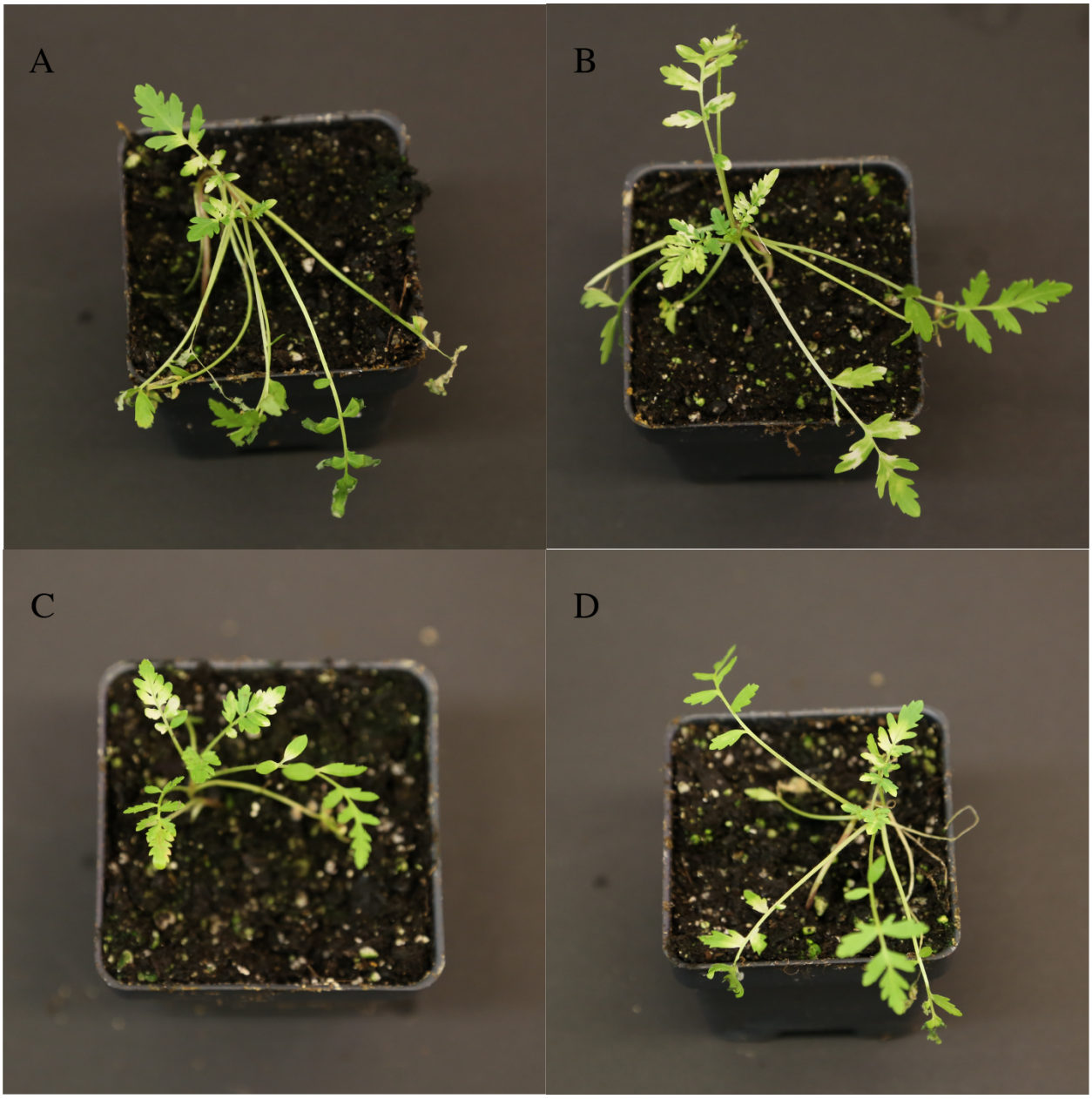
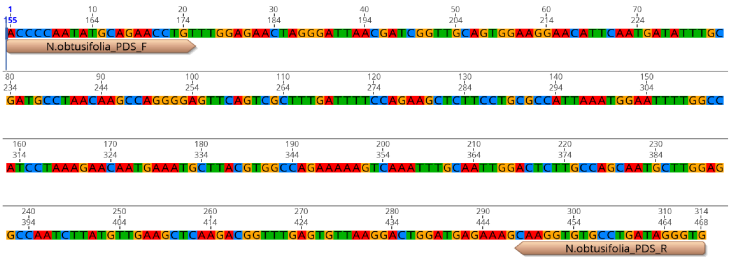


Figure 13. *PDS* silencing of VIGS experiments of *Schizanthus grahamii*. Phytoene desaturase (*PDS*) (A-D). *PDS* was successfully down regulated in *Schizanthus grahamii*. Plant showed lighter green leaves in newly developed leaves 3 weeks after the infiltration.

Phytoene desaturase (PDS)



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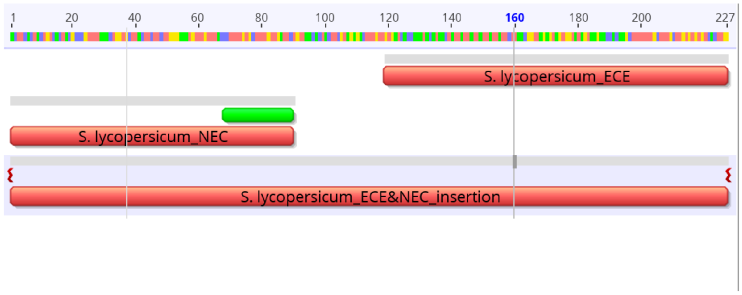

So. lycopersicum CYC2A

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AGAGACTCAAGGGCAAAGGCTAGAGCAAGAGC

So. lycopersicum CYC2B

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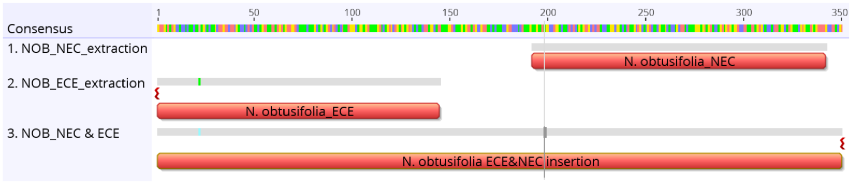

N. obtusifolia CYC2A

GGCTATTCACAAAGTCCAAATTAGCCATTGAAGAGCTCACTACTGCTCAGATCAAAATCACCAGCCCTAATGAGTCGTCAGCAGCAAAGAAAAGTCCTTCATCTATTAAT---TCAGAAT
GTGAGGACGTTGTTCTTGCAAGAGCAAAAGAAGAGGAGGCTATACTTCATTTGATTGCGAGAGAGTCCA

N. obtusifolia CYC2B

CCTAGCAAAACCCTCGATTGGCTTTTCACTAACTCGAAACTAGCCATTGAGGAGCTCATTAATTGGTCCACTCATCAGGATCATCACCCAAAGATTGCAGGAGCAACGAAGTCGAGC
TGCAATGAGACCGCCAAGGATTGTGCT----------TCACAGTGTGAAGACCTGGCC----------ATAACAACAAATGAGGGTTCGGAAAGA

Figure S1. Sequence alignments and primers used for the VIGS experiments. Sequences were analyzed and identified *CYC2A* and *CYC2B*. Primers were designed using sequence of both *So. lycopersicum* and *N. obtusifolia* (RED) to create fragment for TRV2 vectors.

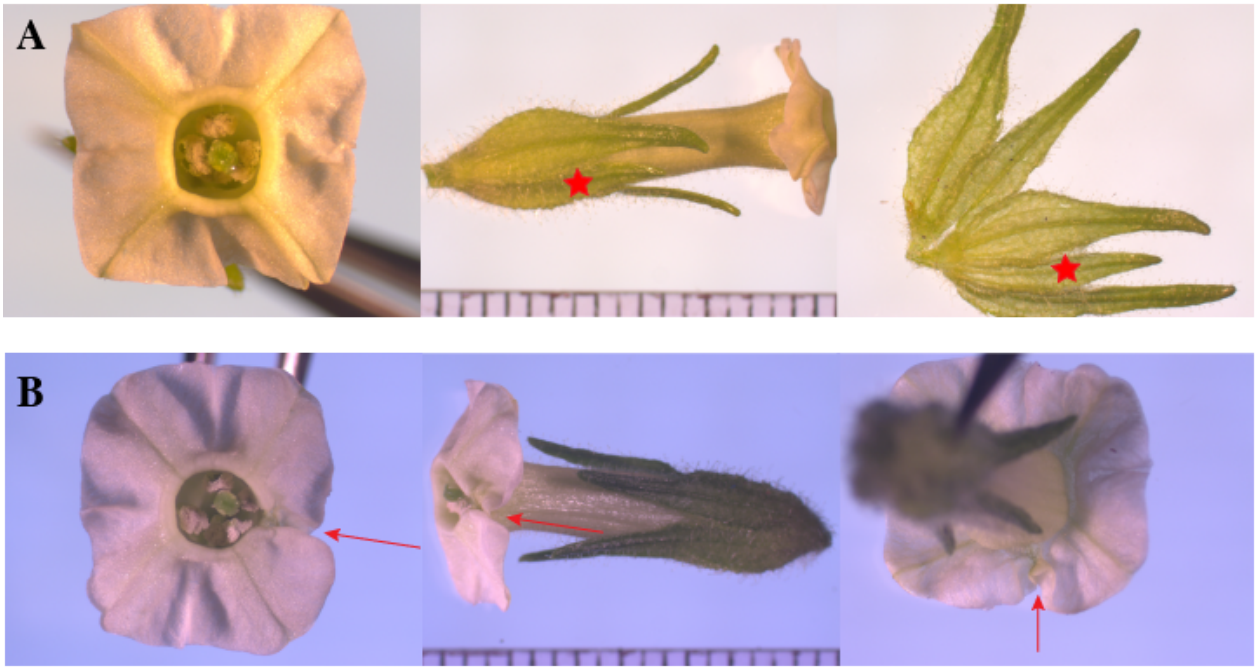

Figure S2. Separated petal and shortened sepal of flowers observed VIGS *CYC2A;CYC2B* (A-B) of *N. obtusifolia*. Two of *CYC2A;CYC2B* silenced *N. obstuillia* flowers that showed separated petal and shortened sepals. (A) One of the lateral petal was not completed aborted and was separated. (B) flower showed 4 petals and stamens, however, one of the lateral petal was not completed aborted.

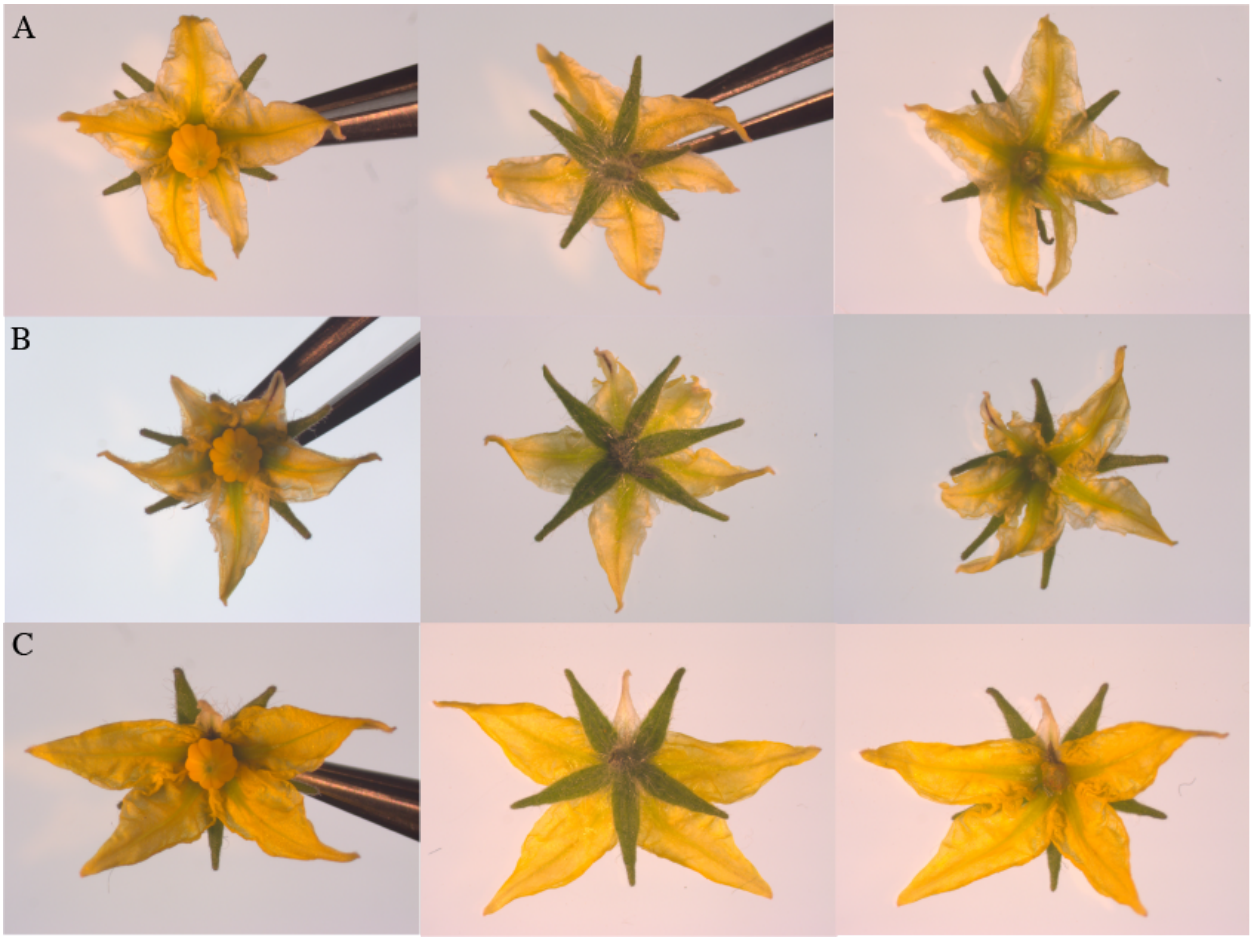

Figure S3. Floral phenotypes including possession of additional sepal (A), aborted petals (B and C) observed in different plants of VIGS *CYC2A;CYC2B* of *So. lycopersicum*.

(A) A flower showed additional sepal while there is no change of the petal number. (B) The aborted petal of a flower shows the black mark on midvein, and the orientation of sepals is also shifted. (C) A flower shows an aborted petal while the sepal orientation of the wild type is maintained.

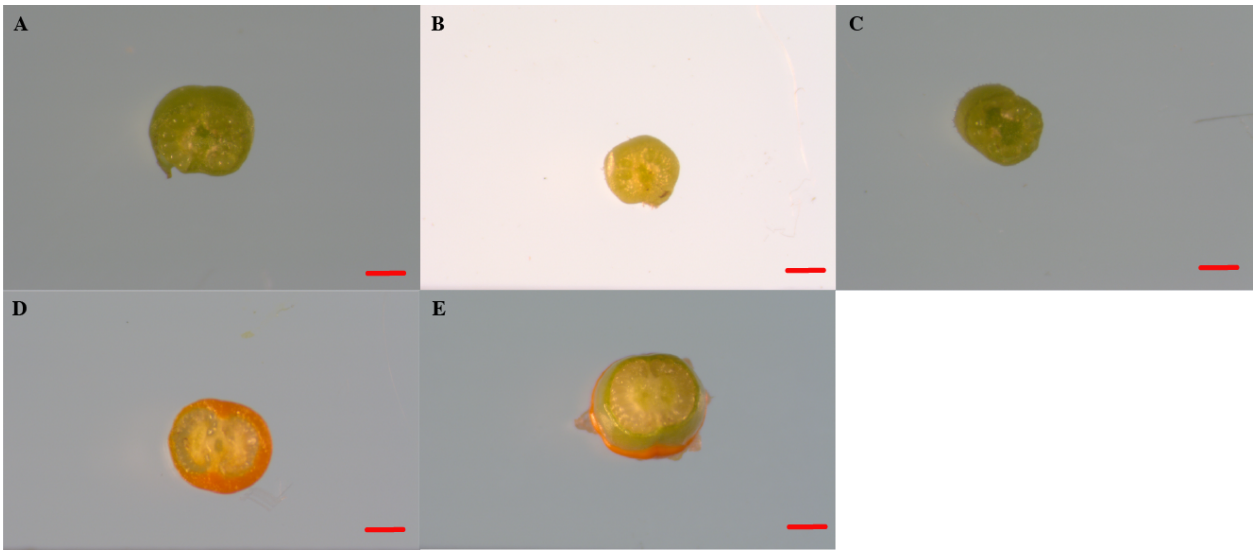

Figure S4. Carpel and ovule morphology of wild type (A), empty vector (B), and VIGS *CYC2A;CYC2B* (C) of *So. lycopersicum* and wild type (D), VIGS *CYC2A;CYC2B* (E) of *N. obtusifolia*. There is no morphological differences were observed among wild type (A), empty vector (B), and VIGS *CYC2A;CYC2B* (C) of *So. lycopersicum*. Also in *N. obtusifolia*, no morphological differences were observed among wild-type (D) and VIGS *CYC2A;CYC2B* plants. Scale bar = 0.5mm

Table S1. Numbers of plants used for VIGS experiments

	So. lycopersicum		*N. obtusifolia*	
	Initial # of Plants	# of Survivals	Initial # of Plants	# of Survivals
Wild Type	40	33	13	10
Empty Vector	9	8	-	-
PDS	60	56	25	23
CYC2A;CYC2B	55	45	23	21

VITA

Joonseog (Joon) Kim was born on March 30th, 1986 in Seoul, South Korea. Joon received his Bachelor of Science in Biochemistry and Molecular Biology from the Chemistry Department from University of Richmond, Richmond, VA in 2014. Joon entered in the Master of Science graduate program in the Department of Biology at Virginia Commonwealth University (VCU) in 2015, and he will begin PhD studies in the Integrative Life Sciences program in the Fall of 2017 at VCU.