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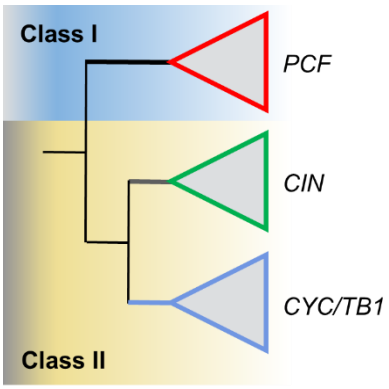
The Functional Diversity of *TCP* genes during the evolution of flowering plants: A Case study of *CYC2*-like genes in *Schizanthus* (Solanaceae)

I. Introduction

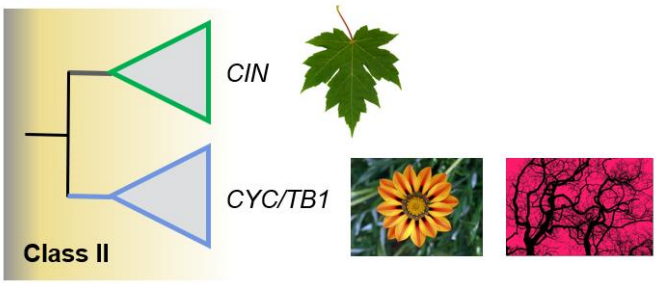
TCP gene family was first described in 1999, as a group of plant genes encoding proteins sharing the TCP domain that allows DNA binding, protein-protein interactions, and encode for transcriptional factors [11]. Teosinte Branched1 (tb1) / Cycloidea (CYC) / Pro-Liferating Cell Factor (PCF 1 & PCF2) (TCP) family is the given family name for these plants specific genes [1]. Tb1 was originally found from maize, CYC was first found from snapdragon, and PCF1 & PCF2 were initially detected from rice. These transcriptional factors greatly influence the growth patterns of tissues and organs during plant development, which are key essentials for plants to form [1].



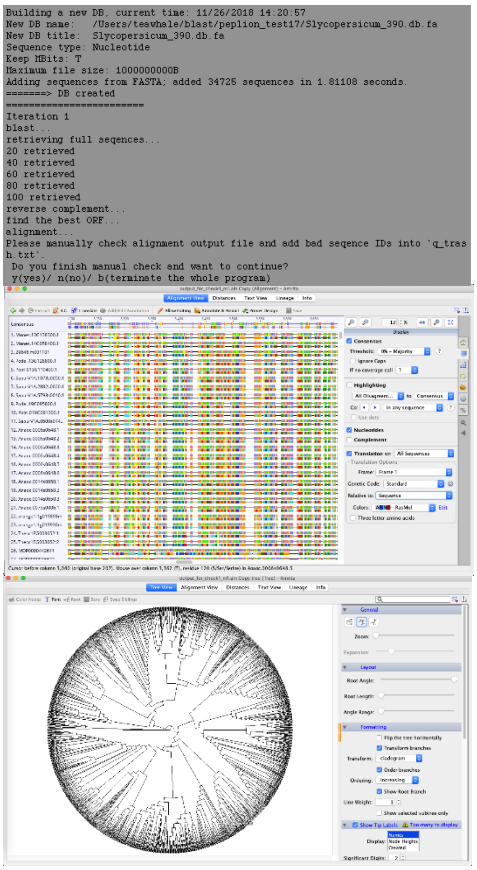
Based on previous gene evolution study, TCP genes fall under two classes known as Class I and Class II [1]. They are distinguishable by four-amino acid deletion in the domain of Class I compared to Class II proteins [1]. Although both classes are given significance to these TCP genes, the gene function of Class II members of the TCP family are better studied. Within the Class II there are two further lineages found known as Cincinata (CIN) and Cycloidea/Teosinte Branched 1 (CYC/TB1) clades [11]. The members of CIN clade has been suggested involving in the development of leaves in multiple plant

species. CYC-like and TB1-like genes are usually found in the control of the floral symmetry and branching patterns, respectively [11].

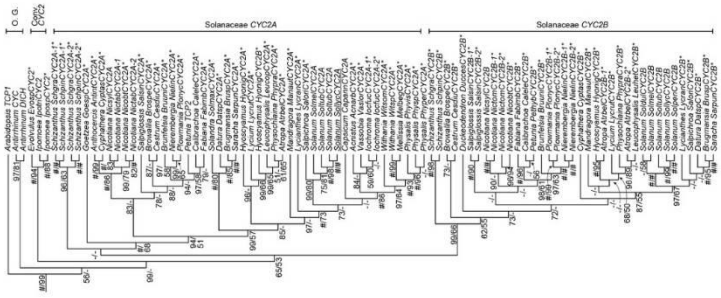
These traits are essential for evolutionary fitness and they affect important aspects of plant ecophysiology such as light interception efficiency, adaptation to resource availability, and pollination success [1].



The last large-scale phylogeny of TCP genes including the species from green algae to flowering plants inferred based Next-Generation Sequencing (NGS) database was published about 11 years ago [2]. Since then, by using the new NGS technology, more NGS datasets (both whole genome and transcriptome data) have been published [7]. Therefore, in this project, we use a newly designed iteration Basic Local Alignment Search Tool (BLAST) to mine TCP genes from these published Next Generation Sequencing (NGS) database thoroughly from about 80 different plants [12]. The NGS database was processed by Blast once runpip.sh file runs which it then retrieves sequences from this



database, takes the reverse compliment, finds the Open Reading Frame (ORF), and gets the alignment. After these steps, Geneious software is used to manually check for regions in the sequences that share similarities. Sequences that are significantly different from other sequences are deleted. This process is repeated until no more sequence sharing the sequencing



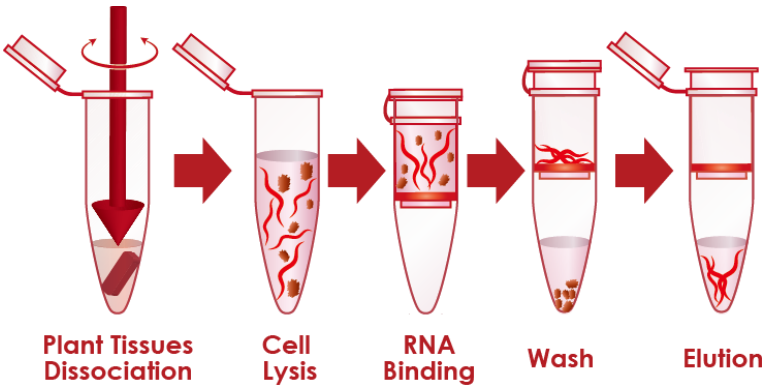
similarities is found. After data mining is performed, we processed the gene evolution study by reconstructing the phylogenetic relationship between the detected TCP genes. When focus on the CYC2 linage of CYC/TB1 clade, we found that the gene duplication and lost are commonly happened during the evolution of CYC/TB1 clade in flowering plant. For example, the family level gene duplication of CYC2-like gene occurred at the family level of Solanaceae. Furthermore, in Schizanthus, one of the CYC2 clade processed a genus-level gene duplication. The gene function of CYC2 genes was firstly well studied in snapdragon and later in several more flowering plant groups, e.g., Fabaceae, Malpighiaceae, and Asteraceae et al. [3]. In these previous studies, CYC2-like genes have been confirmed playing a key role to establish the floral zygomorphy by the dorsiventrally differential expression on the floral meristem [3]. Species of Schizanthus have significant zygomorphic flowers. Therefore, we want to test whether the CYC2-like genes of Schizanthus, that with multiple copies caused by gene duplication events, also involve the development of floral zygomorphy. Virus Induced Gene Silencing technique

is a method that silences a particular gene from being expressed. This method will be used to silence the CYC2-like gene in *Schizanthus pinnatus* and to observe whether the floral symmetry will be modified or not.

II. Experiment

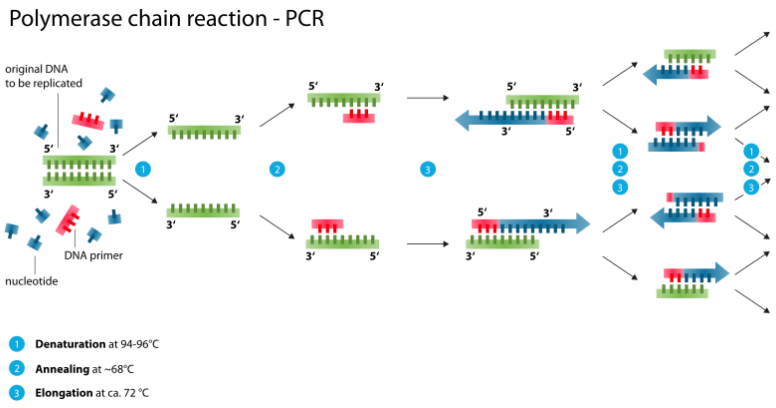
The first step in performing the Virus Induced Gene Silencing Method (VIGS) will be obtaining 20-30 seedlings of *Schizanthus pinnatus* and growing them in pots for about 2 to 3 weeks [4]. They will be kept and maintained in the greenhouse chamber until true leaves developed. Silencing will start in roughly 3 weeks [4]. VIGS will have a lower silencing efficacy performing on plants older than 4 weeks [4].

The second step will be undergoing RNA extraction. This is when an appropriate tissue of a full grown wild-type Schizanthus will be selected to extract all the RNA sequences. In order to do this process, plant tissues will be put into a test tube for dissociation with a pestle several times [15]. This will break all the plant tissues into smaller pieces. Then lysis buffer will be added to break all the cells, which will extract RNA strands as well as small molecules and ions [15]. RNA strands and



these molecules will be transferred to another test tube that contains a filter so that the RNA can bind to it [15]. All the liquid with those small molecules will then be drained. Finally, when all the RNA is obtained, it will be then be transferred into another test tube to undergo elution, which will wash down RNA even further [15]. RNA will then undergo reverse transcription to become complementary DNA (cDNA), which will then be used for Polymerase Chain Reaction (PCR).

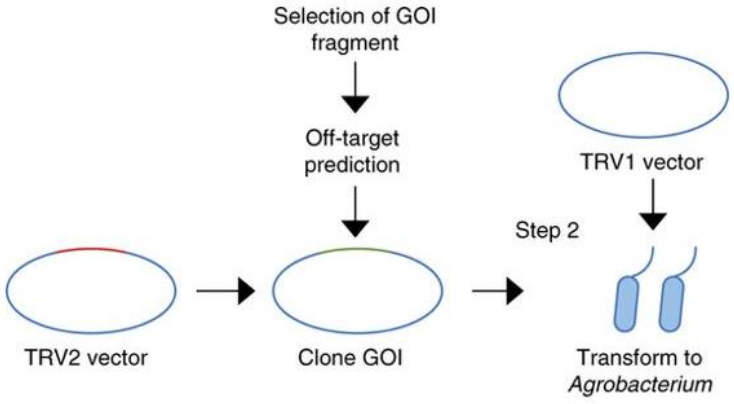
Polymerase Chain Reaction is the third step of VIGS, which is basically a method that creates multiple copies of fragments of a desired gene from the DNA that is extracted [14]. The goal is to obtain multiple copies of CYC2 gene fragment that was found in NGS database through Blast. The complementary DNA that will be amplified is mixed with deoxyribonucleotides, a thermal stable DNA polymerase called TAC polymerase, and DNA primers [14]. Based on the CYC2 gene fragment that was selected from NGS



database, the first 15-10 nucleotides that make up DNA primers for CYC2 gene fragment specifically, will be selected and given to the Integrated DNA Technologies (IDT) company for creating the DNA primers. The DNA primers will be hybridized to the ends of the gene to be amplified and will provide a starting point for the tack polymerase [14]. The mixture will be heated to

break the hydrogen bonds in the DNA forming single-stranded molecules [14]. Then the mixture will be cooled to allow the DNA primers to anneal each end of the segment to be copied [14]. TAC polymerase will then synthesize the complementary strand of the DNA using the DNA primer as the starting point [14]. This process will be repeated until enough CYC2 gene fragments are produced for the next step of VIGS. CYC2 gene fragments will produce siRNAS with a minimal predicted off-target gene silencing [4]. About 200 – 400 nucleotides will be selected from a coding or an untranslated region (UTR) of the CYC2 gene [4]. Poly-A-tail sequences will be avoided.

The fourth step of VIGS will be preparing the infiltration solutions. There are many vectors used in VIGS that are available for gene silencing in various plant species, however, there are only 22 commonly used that are capable of silencing genes in more than one plant species [4]. Two most used vectors known as Tobacco Rattle Virus 1 (TRV1) and Tobacco Rattle Virus 2 (TRV2) are used in Solanaceae family species to silence targeted genes [10].



There are many other VIGS vectors that can be used, such as PVX-based vectors, however, TRV1 and TRV2, as a bipartite vector set, has been confirmed that can effectively silence a target gene [9]. It can also infect meristem tissues while other

vectors cannot. For example, if PVX-based vectors were used, then it can create symptoms such as leaf distortion and localized cell death, or it can make it difficult to interpret the silencing phenotype [4]. Compared to PVX vectors, TRV vectors can show milder symptoms instead [4]. The role of TRV2 vector is to contain the CYC2 gene fragment while TRV1 vector will help TRV2 to deliver the CYC2 gene into Schizanthus [4].

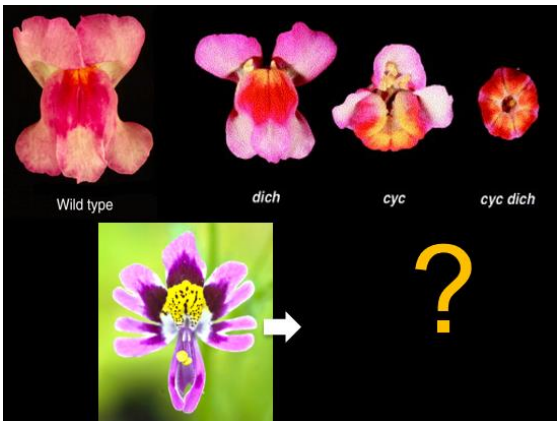
Each TRV vector will be transferred into Agrobacterium, separately [4]. Agrobacterium is used for its ability to transfer DNA between itself and plants, which is a method that is efficient, easy, and cost-effective compared to direct inoculation of the virus particles [13]. After putting the TRV vectors into the Agrobacterium, the Agrobacterium will be grown overnight [4]. The Agrobacterium containing TRV1 and TRV2 vector will then be mixed within the infiltration solution [4], which will be used for the final step of VIGS.

The final step of VIGS is to infiltrate Schizanthus in order to silence the CYC2 gene. Infiltration can be done using common methods, such as using toothpicks, spraying, syringing on leaves, needle syringing, and vacuum infiltration [5]. Vacuum infiltration will be chosen for this procedure as it is more efficient to infiltrate the whole species to silence the CYC2 gene, and to get accurate results rather than injecting in one part of the area. For this procedure, the entire species will be submerged into a beaker

container the infiltration solution, and then the beaker is placed in a vacuum chamber [8]. The vacuum will be able to force the air out of the intercellular spaces within the leaves [8]. When the vacuum is released, the pressure difference forces the Agrobacterium suspension into the leaves through the stomata into the mesophyll tissue [8]. The whole plant will be contacted with the bacteria and the vector inserted with CYC2 gene will be transferred [8]. This procedure should result in silencing the CYC2 genes from being expressed.

III. Discussion

In previous studies, in snapdragon, when the two CYC2 genes, DICH and CYC were silenced, the zygomorphic flowers became actinomorphic [6]. This suggested that the CYC2 genes played a role for establishing the floral zygomorphy in snapdragon. Therefore, the purpose of this experiment is to silence the CYC2 gene in Schizanthus and



infer any possible gene functions by the morphological differences between the wild type and the mutant type [6]. Silencing the CYC2 gene may result in the changes of floral symmetry of Schizanthus, for example, similar as snapdragon, having an actinomorphic floral phenotype [6]. This is when the species will have a one plane of symmetric pattern rather than multiple planes of symmetry. This result should indicate that the CYC2 genes indeed control floral symmetry as found in snapdragon [6].

Otherwise, it suggests that CYC2 genes may not involve the establishment of floral symmetry in Schizanthus [6].

In terms of any limitations and pitfalls that can occur using VIGS, the CYC2 gene sequence may not be silenced completely through the whole plant. This can lead to a mosaic pattern in the phenotype. In order to fix this, we can process more individuals of Schizanthus to compare results. Another problem that can occur is the limitation on the size of the CYC2 gene fragment. If the fragment is too long, we can use enzymes to cut it into smaller fragments and use them to insert in TRV2. Lastly, the vacuum infiltration step should be done at an earlier stage of Schizanthus. VIGS is more efficient in plants that are 2 to 3 weeks old. If they are older than that, then the infiltration efficiency will be lower that will cause the low silencing rate.

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