Characterizing B- and C-class Floral Organ Identity Genes in a Homeotic Floral Mutant

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Abstract

The 'ABCE' model of flower development explains the patterning of organs along the flower axis. Involving mostly MADS box genes that cause homeotic mutations, this model has been the botanical counterpart to the patterning of the animal body plan by HOX genes. The flowering plant species *Thalictrum thalictroides*, in the buttercup family Ranunculaceae, belongs to an interesting lineage that is sister to all other eudicots, including the model system *Arabidopsis thaliana. T. thalictroides* flowers consist of three types of organs; petaloid sepals, stamens and carpels. I will be using a forward genetics approach to study a horticultural mutant of *T. thalictroides*, 'Betty Blake.' Its phenotype consists of multiple whorls of non-petaloid (green) sepals and multiple whorls of carpels, resembling B-class *Arabidopsis* mutants. We hypothesize that the reason for the extra carpels and extra, non-petaloid sepals in 'Betty Blake' is either a mutation in one of the B-class genes or a change in the regulation region of the C-class gene *ThtAGAMOUS1*. The molecular dissection of this mutant will contribute to understanding the degree of conservation of stamen and carpel identity genes across divergent flowering plants, and test whether there is sub-functionalization of gene function for petaloidy of sepals.

Literature Review

Angiosperms, or flowering plants, are one of the most successful and diverse groups of organisms on the planet, with over 250,000 different species naturally present in the wild (Krizek and Fletcher, 2005). Their success is in part owed to their unique reproductive structure, the

flower, which typically consists of four whorls of organs: sepals, petals, stamens and carpels. The process of floral organ specification is controlled by a set of developmental genes that are necessary for the resulting floral morphology. The evo-devo approach states that alteration to the structure and regulation of developmental genes are responsible for morphological changes. These morphological changes can be the subject to evolution by natural selection, as species with more advantageous (adaptive) morphologies have a higher chance of reproducing. Therefore, the evolution of morphology is ultimately caused by to the evolution of the developmental genes.

The classical 'ABCE' model of flower organ identity links the development of the four floral organs: sepals, petals, stamens, and carpels, to four gene classes, A, B, C and E (Coen and Meyerowitz, 1991). The basic model states that A-class genes determine sepals; the combination of A-and B-class genes determine petals; the combination of C-and Bclass genes determine stamens; and the C-class genes results in carpels. E-class genes must also be expressed in all four floral organs in order for proper development to occur (Ditta *et al*. 2004).

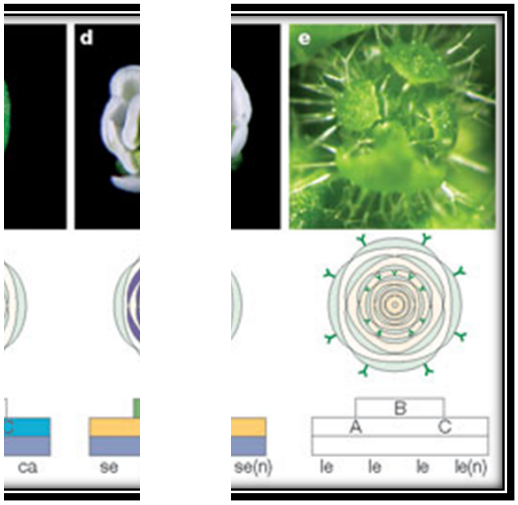

Fig 1. Homeotic floral mutants of *Arabidopsis* mutants and the corresponding 'ABCE' models showing the missing expression for each of different classes (Krizek and Fletcher, 2005).

The model was created after genetic studies of *Arabidopsis thaliana* and *Antirrhinum majus,* two distantly related model species with distinct floral morphologies (Coen and Meyerowitz 1991). A very important outcome of the formation of this model was the conclusion that even though *Arabidopsis* and *Antirrhinum* are phylogenetically distant, the floral developmental genes were homologues in each species. For example, the *Arabidopsis* B-class gene *APETALA3* (*AP3)* is homologous to the *Antirrhinum* gene *DEFICIENS* (*DEF*), (Coen and Meyerowitz 1991). The 'ABCE' model has since been used as guidance to study a wide variety of floral genes. The model increases in complexity when each gene class is broken down into the individual genes. In *Arabidopsis,* there are two B-class genes, *APETALA3* and *PISTILLATA(PI).* (Coen and Meyerowitz 1991). In addition, the number of genes in each gene class can vary from species to species. While *Arabidopsis* only has two B-class genes, the model system of our study, *T.thalictroides* has four; *ThtAPETALA3-1*, *ThtAPETALA3-2a*, *ThtAPETALA3-2b* and *ThtPISTILLATA* (Kramer *et al.* 2003).

The manner in which the protein products from the 'ABCE' model function can be illustrated by the morphologies observed in two *Arabidopsis* mutants: *apetala3* and *pistillata.* These mutants are the result of genetically knocking out either *AP3* or the *PI,* respectively. (Coen and Meyerowitz 1991). Both mutants lack the whorls containing stamen and petals, which are instead replaced by carpels and sepals, respectively (Bowman *et al*. 1989). The fact that knocking either gene out gives the same phenotype can be explained by the Floral Quartet Model. The model explains how the development of each floral organ requires the protein products of the 'ABCE' genes to form protein tetramers in order to function (Theissen 2012).

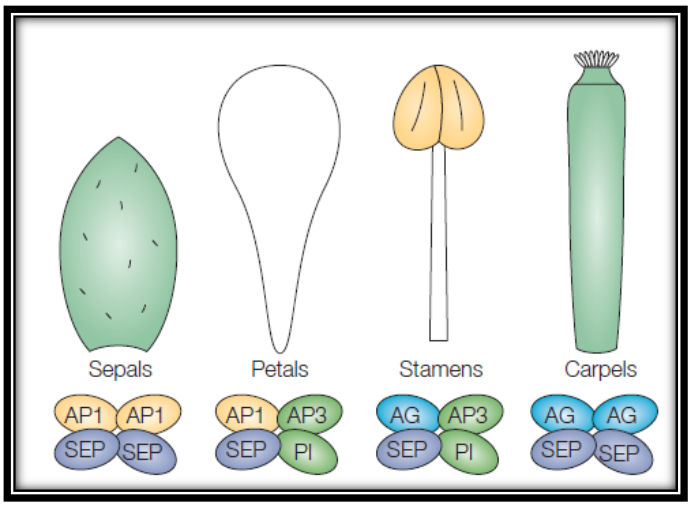

Fig 2. The Floral Quartet model explains how 'ABCE' protein tetramers, are need for the development of floral organ.

According to this model, the two B-class proteins, *AP3* and *PI* are both necessary for the formation of petals and sepals in *Arabidopsis* (Krizek and Fletcher 2005). Therefore even though the mutants lack only one of the B-class genes, the protein tetramers for petals and sepals are not complete, causing a complete loss of B-function and homeotic conversion of stamens and petals to sepals and carpels.

All of the 'ABCE' genes contain regulatory regions that are necessary for the correct gene expression. The master regulator of floral identity in both *Arabidopsis* and *Thalictrum* is the gene *LEAFY* (*LFY*). *LFY* binding sites are known to be present within many of the floral identity genes. Along with the *LFY* motif where the *LFY* binds to, there are other cis-regulatory element motifs present in the regulatory regions of these genes. These motifs, such as the CArG box, help regulate the genes by binding other transcription factors that may act as activators or repressors (Causier 2009).

Introduction

Thalictrum thalictroides, commonly known as rue-anemone, is a species of plant from the buttercup family native to the woodlands of Eastern North America. Instead of the typical four whorls of flower organs, *T. thalictroides* only has three: sepals, stamens and carpels. The sepals are unlike typical sepals which display a green leaf like morphology, instead the *T. thalictroides* sepals exhibit a morphology similar to petals. Petals are classified as being large and showy, non-photosynthetic (white or colored), having a narrow base, and are located in the second whorl, inside of the sepals and outside of the stamens. The sepals found in *T. thalictroides* are large, white or pink, and are located in the first whorl. This leads to an interesting situation when studying one of the mutants of *T. thalictrodies*, a cultivar known as 'Betty Blake.' Unlike wild type, 'Betty Blake' only has two distinct floral organs: sepals and carpels. The sepals found in 'Betty Blake' exhibit a green, leaf-like, morphology more commonly seen in sepals, rather than the petaloid morphology found in wild type.

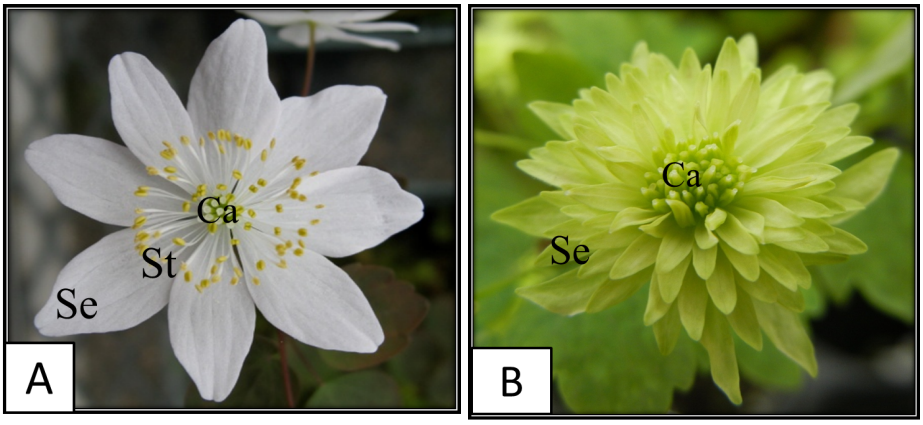

Fig 3. Phenotype of *Thalictrum thalictroides*. (A) Wild Type has petaloid sepals, stamens and carpels. (B) 'Betty Blake' has multiple whorls of non-petaloid sepals, and carpels.

A likely cause for the converted carpels and non-petaloid sepals in 'Betty Blake' could be one or more dysfunctional B-class genes (*ThtAP3*'s or *ThtPI*) or a mutation within the regulatory

region of intron one in *ThtAGAMOUS1 (ThtAG1).* The coding region of *ThtAP3* and *ThtAG1* genes have been sequenced and aligned to the coding region of wild type. No mutations were found in the coding region of these genes. Therefore we hypothesize that 'Betty Blake' has a regulatory mutation in a B- and/or C-class gene.

Hypothesis 1: One or more of the B-class genes, *APETALA3* (*ThtAP3-1, ThtAP3-2a, ThtAP3-2b)* and/or *PISTILLATA* (*ThtPI*), could be dysfunctional in 'Betty Blake.'

Hypothesis 2: 'Betty Blake' could be a weak C-class mutant, with a mutation within the regulatory region of the gene *ThtAGAMOUS1* (*ThtAG1*), that alters its expression domain.

Methods and Protocol

Plant Materials

T. thalictroides wild-type and the 'Betty Blake' cultivar were purchased from nurseries and grown in the University of Washington greenhouses.

Sequencing of the *TthAP3-1* genomic locus for 'Betty Blake' and Wild Type

The complete 'Betty Blake' *ThtAP3-1* locus was PCR (Polymerase Chain Reaction) amplified and sequenced from genomic DNA. The PCR master mix for a single reaction contained; 18.2 μl of miliQ water, 2.5 μl of 10Xbuffer, .7 μl of $MgCl_2$, .5 μl of dNTPS, 1 μl for the DNA template, and 1 μl of "Invitrogen Platinum Taq." The PCR was programmed with an annealing temperature of 55^{o} Celsius for 35 cycles (55X35). The primers used on *ThtAP3-1* primers were *TdAPETALA 3*-1 Forward whole coding 5'- ATG GGG AGA GGA AAG ATT GAG ATC AAG -3.' And *TdAPETALA3-1* reverse whole coding, 5'- TCA ACC TAA TCG AAG ACC CTC GAA TCC -3.' Gel electrophoresis was used to separate and confirm the size of the PCR products. The gel mix was made by boiling .6 grams of agarose and 50ml of TAE buffer, then mixing it and repeating once. 4 μl of ethidium bromide were then added, after the mix had cooled.

After confirming the PCR product with the gel electrophoreses, the 'Betty Blake' PCR product cleaned using the ExoSAP-IT protocol, then direct sequenced through Gene-Wiz. The wild type *ThtAP3-1* genomic locus, was first cloned using the "Invitrogen TA Cloning Kit" protocol then sent to be sequenced. Once the sequences of the DNA were obtained, they were placed in a contig along with the wild type coding region sequence, and the genomic sequence from *Aquilegia coerulea AG1*, in Sequencher (v. 4.9). The *A. coerulea*, *AG1* sequence was obtained from the online database Phytozome. Afterwards, the contig was hand-aligned using the software programs McClade and Mesquite.

Analyzing *AGAMOUS1* in 'Betty Blake' and Wild Type

Previously amplified sequences of *ThtAG1* intron one from 'Betty Blake' and wild type were analyzed using alignment software. Sequencher (v. 4.9) was used to create a contig of the both sequences. Then Mesquite and McClade were used to hand align the sequences and verify the locations of the motifs, using their search feature.

Results

The sequenced genomic, sequence of *ThtAP3-1* from 'Betty Blake,' was approximately 1.5kb. When compared to the coding region of *ThtAP3-1* found in wild type and an ortholog of *AP3-1* in *A. coerulea*, a related species with a published genome, the alignment allowed us to identify and locate the size of 6 introns.

The *ThtAG1* sequence for wild type and 'Betty Blake' has been sequenced and has been aligned. However unlike *ThtAG1* for wild type, which has been completely sequenced. The sequence for 'Betty Blake' is only complete up to the second exon. From the alignment, all motifs identified in wild type were found to be identical in 'Betty Blake.' In total three CCAATCA motifs, three aAGAAT motifs, two *LFY* binding sites, and three CArG-box motifs were identified in the same location in both wild type and 'Betty Blake' (Causier 2009.)

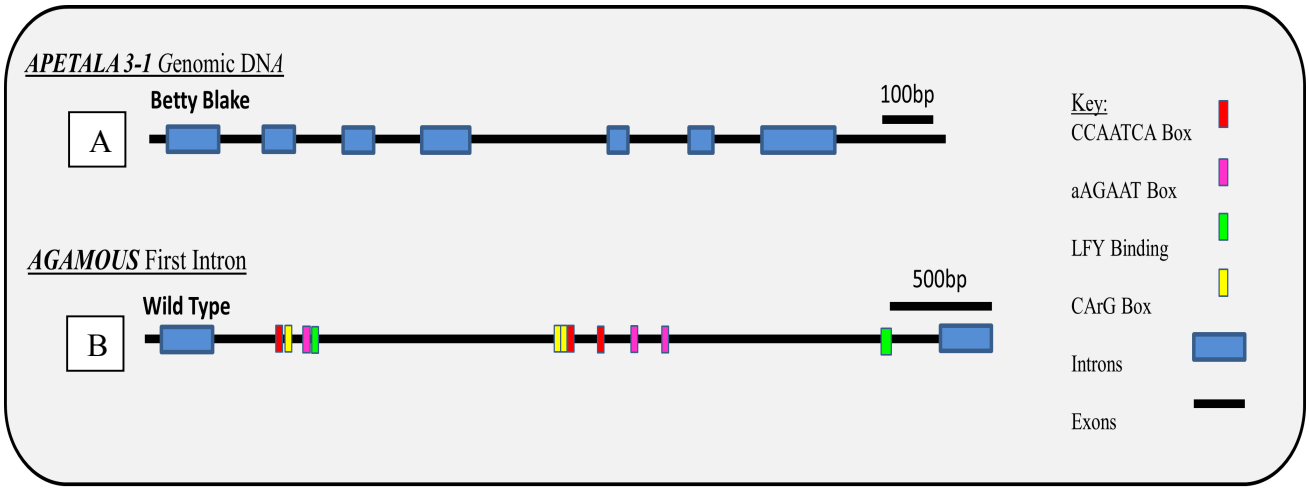

Fig 4. (A) Gene schematic of *TthAP3-1* in the 'Betty Blake' mutant. The total length is approximately 1.5kb, and contains 7 exons and 6 introns. (B) Schematic of wild type *ThtAG1* from the beginning of the first exon to the end of the second exon. Multiple regulatory motifs needed for correct gene function are indicated. The location of the regulatory motifs in *ThtAG1* intron one for 'Betty Blake' are identical.

Discussion

By aligning the genomic *TthAP3-1* sequence from 'Betty Blake' to the coding region of the wild type we were able to identify the length and the locations of the introns. Since we lack the genomic sequence of wild type we cannot exclude the presence of mutations within the introns. These mutations are likely candidates for explaining the phenotype of 'Betty Blake.' The 'Betty Blake' *TthAP3-1* genomic sequence, was also aligned with an ortholog of *AP3-1* in *A. coerulea.* This alignment revealed similarities within the gene structure which allows us to conclude that it is unlikely that the *ThtAP3-1* gene contains a large mutation that would cause the phenotype of 'Betty Blake.' However the small mutations could be present within the gene and may potentially have some effect on the gene which could explain 'Betty Blake.'

When the *ThtAG1* gene of *T. thalictrodies* was aligned with the mutant, no difference in the four cis-regulator element motifs were found, within the first intron. The locations of the CCAATCA box, aAGAAT box, *LFY* binding, and CArG box were all in the same location for

both wild type and the mutant. This allows us to supports conclusion that it is unlikely for ‘Betty Blake’ to be a C-class mutant. Since the largest regulatory region is found within the first intron of *ThtAG1,* and no mutations were found, then the chances of regulatory mutations in other areas of *ThtAG1* are slim. However the complete genomic sequence for ‘Betty Blake’ *ThtAG1* is needed to completely prove or disprove the possibility of ‘Betty Blake’ being a C-class mutant.

Furthers studies should involve sequencing the remaining B-class genes (*ThtAP3-2a, ThtAP3-2b* and *ThtPI)* as they could hold the answer to ‘Betty Blake,’ mutation. These three genes could contain mutations within the regulatory regions within the introns, that could cause them to function improperly. It will also be necessary to sequence the remainder of the C-class gene *ThtAG1* as it could also contain mutations within the regulatory region that would explain ‘Betty Blake.’ The entire genomic *ThtAG1* in ‘Betty Blake’ has yet to be sequenced. Therefore any other mutation within the regulatory regions could hold the answer.

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