Investigating KNOX Gene Expression in Aquilegia Petal Spur Development

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Summary

Organ growth in many flowering plants progresses through two phases: cell proliferation and cell elongation. Recent work has indicated that class 1 KNOX genes regulate cell proliferation. These genes encode transcription factors that are largely responsible for a steady supply of undifferentiated stem cells. Moreover, KNOX gene expression is detectable in lateral organs of different plants such as compound leaves, suggesting a role in sculpting organ shape. The association of KNOX genes with stem cells suggests a role for these genes in the cell division phase of plant development. This suggests that a specific lateral organ, petal spurs, may be built in part using KNOX genes. Indeed, previous work has shown that KNOX gene over-expression in petals may cause spur-like outgrowths. In Aquilegia, a short proliferation phase gives rise to nascent spurs, which is followed by a cell elongation phase. We investigated KNOX gene expression and found by using Reverse Transcription Polymerase Chain Reaction, that expression of KNOX genes was detectable, but not uniformly so, suggesting that there may be either a highly cell-specific expression of KNOX genes or extremely low expression in petal spurs. Future tests with micro dissected tissue samples may prove to be helpful but it can be concluded for now that KNOX genes are not highly or consistently expressed in Aquilegia petal spurs, and their role in this organ is an ongoing mystery.

Introduction

Whether or not similar genetic pathways are used to program convergent biological structures poses an interesting evolutionary question. Plants, lacking cellular mobility, are particularly adept at co-opting regulatory pathways and assigning them new roles, resulting in novel structures. For example, the pathway that maintains undifferentiated stem cell fate and then promotes differentiation into lateral organs in the shoot is deeply conserved within plants. At the same time the genetic players have been co-opted several times independently to function in new spatial or temporal areas, resulting in different patterning of lateral organs (1).

Petal nectar spurs have evolved several times independently across the flowering plants, often times co-evolving with different pollinators (2). The genus Aquilegia is a flowering plant that is a useful model system for genetic and genomic studies (3). The petals remain short for a period during development before extending a blade that curves as it elongates, forming a cup-shape that elongates into the spur (4).

All plant shoot tissue is derived from the apical meristem (shown in Figure 1), which is comprised of a region of undifferentiated cells that can give rise to any somatic tissue. The cells of this region perpetually divide, providing a steady supply of stem cells, which allows the plant to facilitate growth and produce new organs throughout its lifetime. The genetic machinery that is involved in maintaining meristem indeterminacy or in stopping indeterminacy and promoting differentiation can be turned on in other regions of the plant (5).

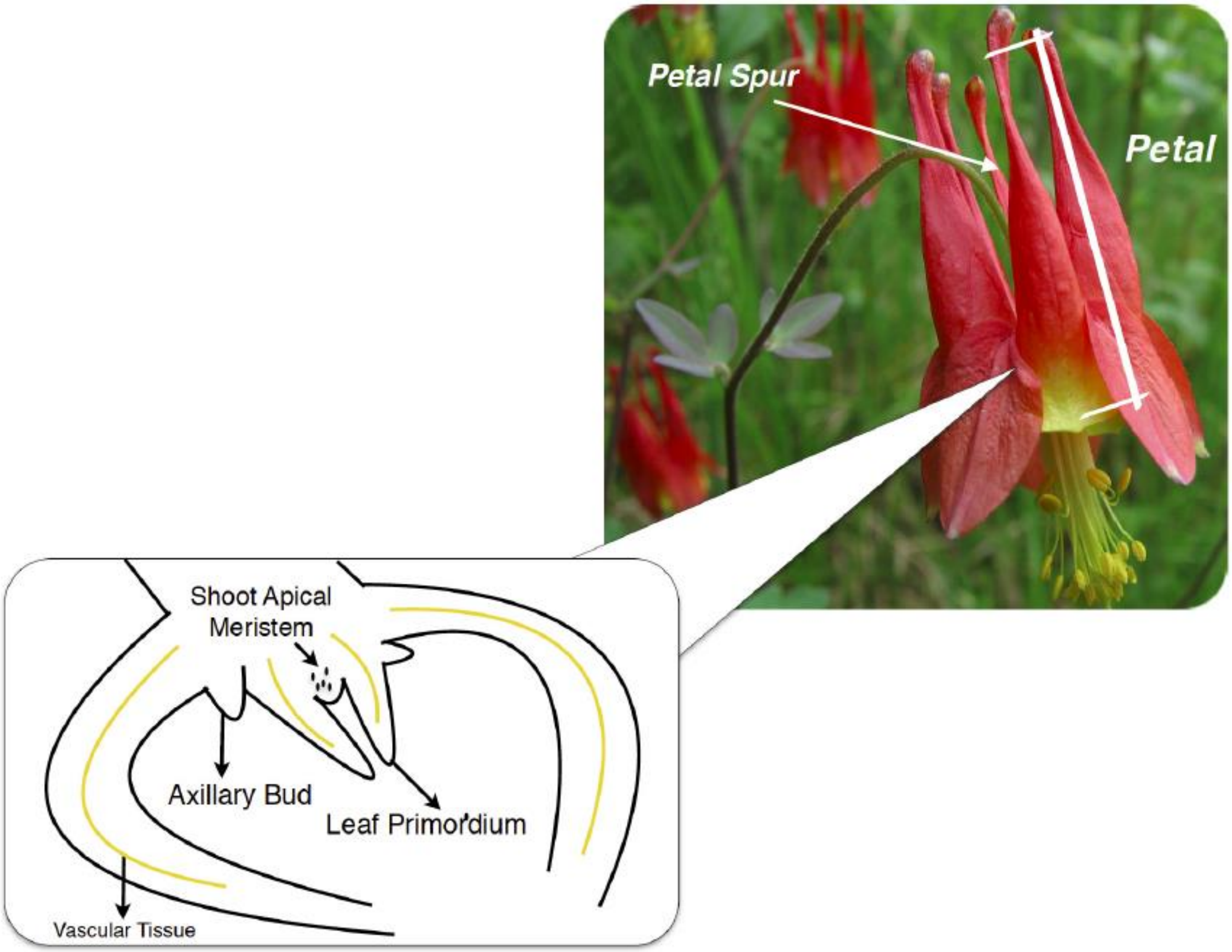

Figure 1. A diagram of Aquilegia caerulea. The shoot apical meristem houses many of the undifferentiated cells for the flowering plant. Just along side it are the leaf primordia, which are lateral outgrowths that eventually develop in leaves. Axillary buds grow from the axil of leaf and may develop into another leaf or even a separate flower. When petals fully develop, they tend to create tubular extensions called spurs, which are evolutionarily significant in terms of pollination. This investigation is

focused on these spurs and by using RT-PCR, we determined which KNOX genes are expressed in this organ. (See 21, 22).

In Arabidopsis, a close relative, there are 8 KNOX genes that are divided into two classes: class I and class II. The class I genes encode four transcription factors: SHOOTMERISTEMLESS (STM), BREVIPEDICELLUS (BP, also known as KNAT1), and KNOTTED-like (KNAT2 and KNAT6) .

Genes such as SHOOT MERISTEMLESS (STM) promote cell proliferation in the shoot apical meristem. These genes encode transcription factors, which are proteins that bind to regulatory DNA sequences of downstream targets. This binding allows KNOX genes to regulate levels of expression of the downstream target genes.

However, KNOX gene function is not limited to the meristems. They have roles promoting compound leaf structure and can partner with another gene family, ARP (8). KNOX genes must be down-regulated in order for cells in the leaf to differentiate, but if they are reactivated in leaves, this results in different areas of the leaf or leaflets. Studies show that in Arabidopsis, expression of class 1 KNOX genes in leaves induces formation of compound leaf structure or leaflets (9). Simple leaves showed high levels of KNOX gene expression in the meristem and no expression in the site of leaf initiation. After induction of KNOX expression, leaves developed with complex leaf phenotypes and various shapes. This shows that there is some relationship between KNOX genes and their partners, such as ARP, in the development of leaf structure as well as with meristem identity.

If KNOX genes can manipulate the growth of these two organs, they may able to influence the development of other organs, such as petal spurs. Spurs are tubular extensions that grow off of floral organs. They have evolved several times independently across flowering plants. It has been noted that there is a distinct correlation between the highly variable length of spurs in the plant Aquilegia and pollinator tongue lengths (10). These outgrowths allow for coevolution with pollinators in the environments, which facilitate rapid speciation among families of flowers (11). For the plant, it would be very efficient to co-opt already present mechanisms for the rapidly evolving organs.

But how would KNOX genes play a functional role in the development of spurs? Let’s start with how plants generally develop. There are typically two stages of cell growth: cell proliferation and cell elongation (12) . The process begins in the apical meristem, in which stem cells divide and give rise to daughter cells. However, if these cells are outside the zone of influence of KNOX genes, they are recruited to differentiate into lateral organs. Lateral organs include leaves or floral meristems if the proper environmental cues are present. These floral meristems then give rise to floral organs.

Spur growth could be due to reactivation of KNOX transcription factors in petals, which may increase the number of cells in the spur. However, this seems unlikely in Aquilegia, as there is evidence of spur development that is void of any KNOX gene expression (11). Furthermore, while expression of KNOX genes in Arabidopsis showed leaf phenotypes analogous to compound leaves, there were no floral phenotypes (13).

Other groups have investigated whether or not the KNOX genes that promote stem cell fate in the meristem have been turned on again in petals to prolong cell division and promote extension of the petal into an elongated organ. The role of KNOX genes could be different in plants lacking spurs. Antirrhinum, better known as snapdragon, does not naturally grow spurs (14). Increased expression of two of its KNOX genes resulted in odd spur-like growths that developed off of the petals. Studies executed in Linaria, a plant that is closely related to Antirrhinum, demonstrated that KNOX genes were highly expressed in the floral organs and the front side of the petals known as the "spur-producing ventral petal" (15). Expression of the Linaria KNOX genes in tobacco resulted in spur-like outgrowths. However, expression of one of the class 1 KNOX genes from the spur producing orchid Dactylohiza fuschii in tobacco failed to produce spur outgrowths (15). These studies indicate that KNOX genes are expressed in petals of some taxa, and that some of these related genes, but not all, promote outgrowths. If the KNOX genes are responsible for spur growth in some taxa, it is not universal. The studies also did not confirm whether the spur like outgrowths were in fact true spurs.

Past research suggests that spur development in Aquilegia consists of two phases: an initial phase of cell divisions throughout, and then a second phase of cell elongation (16). The initial phase of cell proliferation cannot account for the entirety of growth of the plant. Cell elongation accounts for the rest of the increase in size of the plant. Other research suggests that the effects of KNOX genes do not influence petal spur growth in Aquilegia (Elena M. Kramer, unpublished data). In fact, they have almost no association with the first phase of petal spur development, cell proliferation (11). These complex lateral organs may just be anomalies in terms of petal spur curvature.

It has also become apparent that cell proliferation is an extremely short phase in Aquilegia development (16). A comparison was made to Linaria, which have petal spurs as well and results have shown that KNOX gene expression indeed exists during its cell proliferation phase. This may suggest that a different set of genes control these spur outgrowths. However, we wanted to expand this study to a second system with spurs. We repeated the gene expression studies done in Collani et al. with more petal samples. Confirming these results was an immediate goal because it seemed interesting that the development of similar structures in flowering plants are driven by different mechanisms.

In Aquilegia, cell number is irrelevant, and the second phase is responsible for the majority of spur growth. This investigation's purpose is to further our understanding of the role of KNOX genes in Aquilegia. Since cell divisions are a minor part of spur development, we hypothesized that KNOX genes would not have a role in spur growth. Our results were largely consistent and indicated that the KNOX genes likely do not play a significant role in the development of spurs in Aquilegia (Elena M. Kramer, unpublished data). However, we found expression of one related gene of a KNOX gene in petals.

Results

We chose to use RT-PCR to investigate KNOX gene expression in petals because it was a straightforward and fairly quick process (Figure 2) to isolate RNA, synthesize cDNA, and run the PCR. We used five different samples of petals and tested expression of eight different Aquilegia genes: CYCLOIDEA (CYC), HISTONE4 (HIS), KNOTTED (KN), KNOTTED-LIKE1 (KXL1), KNOTTED-LIKE2 (KXL2), SHOOTMERISTEMLESS1 (STM1), SHOOTMERISTEMLESS2 (STM2), and TCP4 (Figure 3). For our gene expression analyses, we pooled five groups of petals from different flowers. We tested expression of housekeeping control gene ISOPENTYL PYROPHOSPHATE:DIMETHYLALLYL PYROPHOSPHATE ISOMERASE2 (AqIPP2) as a positive control (Figure 4).

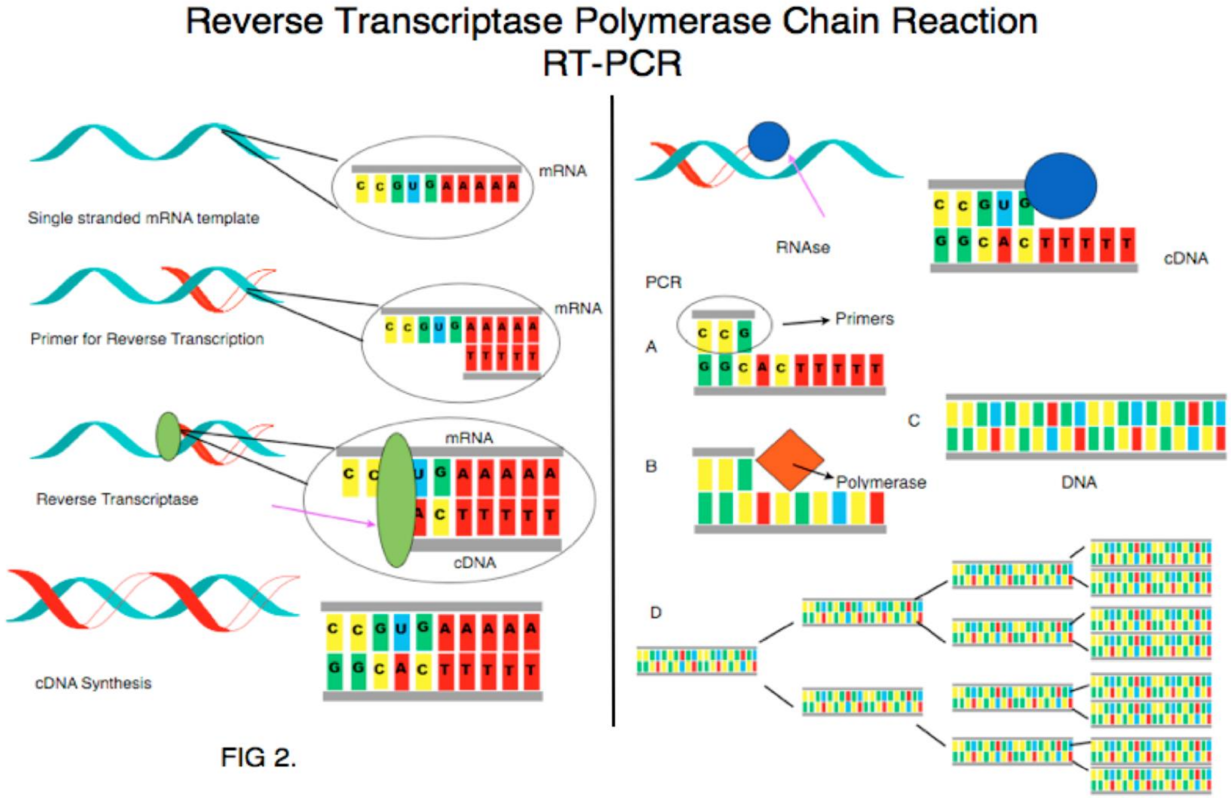

FIG 2.

Figure 2: Overview of Reverse Transcriptase Polymerase Chain Reactions (RT-PCR). Aquilegia RNA was isolated from 5 organs: sepals, petals, stamens, staminodia, and carpels. In RT-PCR, cDNA is first synthesized using KNOX gene primer dilutions, template RNA, and the enzyme reverse transcriptase to add complimentary nucleotide base pairs to the RNA. To initiate PCR, the original RNA template is removed by RNAse and cDNA is amplified. PCR then begins, in which annealing occurs with primers, creating a template for Taq polymerase to complete the double stranded cDNA. Denaturation follows, where primers anneal and extend the cDNA for a set number of cycles, creating amplified cDNA. After the reaction is complete, gene expression can be visualized through stained gels.

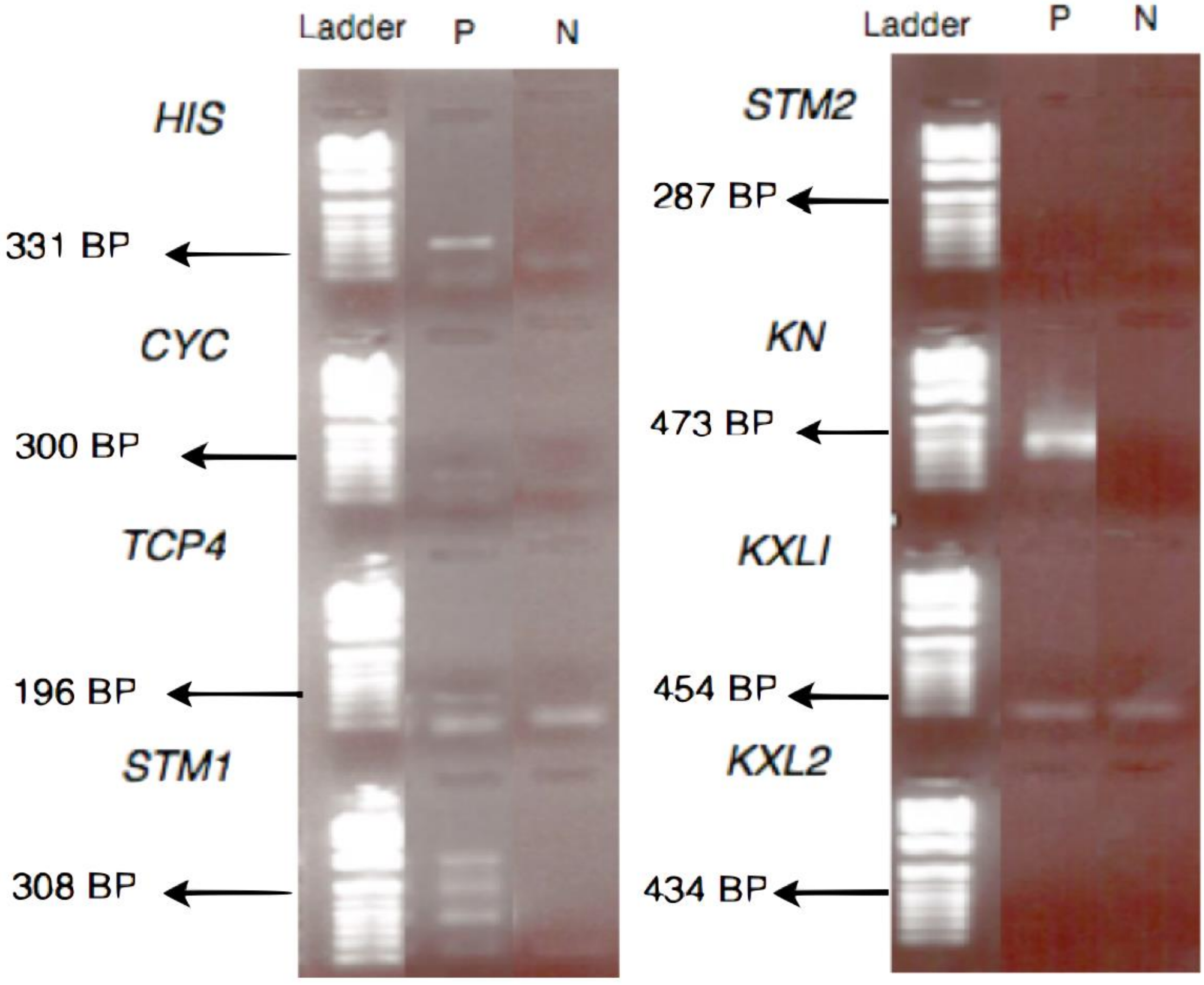

Figure 3. Gene expression in wild type Aquilegia petal spurs.After running RT-PCR, gene expression levels of HIS, CYC, TCP4, STM1, STM2, KN, KXL1, and KXL2 in Aquilegia petal spurs were shown on a stained gel. Results show consistent expression for HIS, TCP4, KN and STM1. However, there is no expression in CYC, STM2 and KXL2. P: petal organ. N: negative control (water). BP: base pairs.

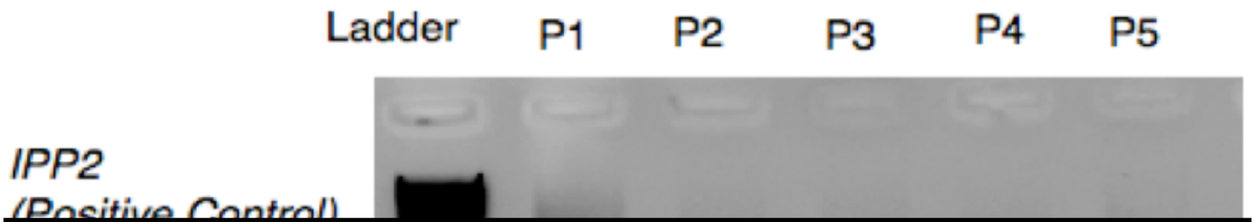

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Figure 4. IPP2 expression in wild type Aquilegia petals. Additionally, RT-PCR was performed on IPP2 and expression levels were shown on a stained gel. IPP2 is a housekeeping gene for Aquilegia, therefore a suitable positive control for the investigation. As expected, IPP2 was highly expressed in 5 different petal spur samples.

HIS4 encodes a known cell division marker (16). KNOTTED (KN) encodes a protein that helps cells perform cytokinesis, a necessary step in cell division. KXL1 and KXL2 are KNOTTED-like homologs identified in Aquilegia (17). STM1 and STM2 are the Aquilegia orthologs of the Arabidopsis STM transcription factor that maintains cell indeterminacy in the shoot apical meristem. CYC is a transcription factor known to have roles promoting petal lobing and has roles in patterning bilateral symmetry (18). Finally, TCP4 is a member of the TCP gene family (19), which consists of several proteins that share a TCP domain. This transcription factor restricts the domain of cell proliferation (20).

The first lane in our gel image (Figure 3) is a 1 kb ladder that was used to check if our band sizes seen from our RT-PCRs were the size we expected from the primers we designed. The second lane shows our petal RT-PCR products from each experiment done with each primer, and the third lane is a negative control, which was performed using water instead of cDNA.

We found that HIS was expressed in petals, which may be an indicator that cell proliferation is active for longer than previously thought. However, TCP4, which acts as an antagonist of cell division, was also moderately expressed. From this we hypothesize that there are different zones of cell division and expansion occurring in our samples. Since we used whole-petal RNA, we hypothesize that HIS might still be active in the blade of the petal, while TCP4 expression is primarily in the spur (Figure 1).

The different KNOX genes that we tested and their corresponding levels of expression raise questions on why there is variation between these genes in petal spurs. From our results, KN seems to be moderately expressed, but we did not see expression of KXL1 and KXL2. We found a small band in KXL1 (Figure 3), but since the band was much smaller than our expected band size of 454 base pairs, and also appeared in the negative control, we expect that this was due to primer dimerization. The expression of KN may imply that cell proliferation occurs in the petal, causing spurs to emerge. It at least indicates that cytokinesis and cell division is happening in some part of the petal.

STM1 and STM2 are both genes that encode transcription factors maintaining cell indeterminacy in the apical meristem. Collani et al. never amplified STM1 at any phase of petal development. Contrary to their results, we amplified a band of our expected size (308 base pairs) in our RT-PCR of STM1 (Figure 3). We also amplified some larger bands. This was not what we expected, as we used the same primers to amplify STM1 that Collani et al. used in their research. The multiple bands could be due to mis-splicing; STM1 is one of several orthologs of the Arabidopsis locus in Aquilegia. The primers may not be as specific as we thought and might be amplifying more orthologs.

In addition, CYC was not expressed at all and this gene has been found to be responsible for promoting petal lobe growth and dorsoventral patterning in Antirrhinum (snapdragon). This implies that the dorsoventral differences seen in Aquilegia are promoted by a different transcription factor.

Discussion

Our results differed from Collani et al. in that while they found expression of KN and STM1 in the inflorescence but not in petals, we saw expression of both of the transcription factors in our petal samples. One can hypothesize a reason for why this is the case. STM in general allows the meristem to produce a ready amount of stem cells. This low-intensity expression could be the cause of a longer cell proliferation phase. These results indicate that further work is necessary to elucidate whether Aquilegia petal spurs are dependent on KNOX genes.

To address these issues in mixed expression levels from these KNOX genes, it may be beneficial to test KNOX expression in different phases of petal development. CYC may be activated in earlier or even later stages than we predicted. To investigate this possibility, RNA extraction of petals spurs in both the earlier and the later stages of development will be required.

We would also like to repeat Collani et al's in situ hybridization and examine gene expression localization of STM1 in Aquilegia petals, also at differing stages of growth. In situ hybridization will help us investigate whether HIS and TCP4 also have differing zones of expression within the petal.

Furthermore, gene expression studies do not confirm if the gene products are necessary for the morphology of the petal. Functional studies such as viral-induced knock-downs of STM1 and KN would be useful to investigate whether impaired function of the protein products leads to petal spur defects.

It is clear that these genetic modules have extremely dynamic expression patterns. KNOX genes remain puzzling regulators of any plant, and to understand more about their function, more extensive tests must be performed that will allow us to obtain increasingly clear results.

Methods

Plant Material

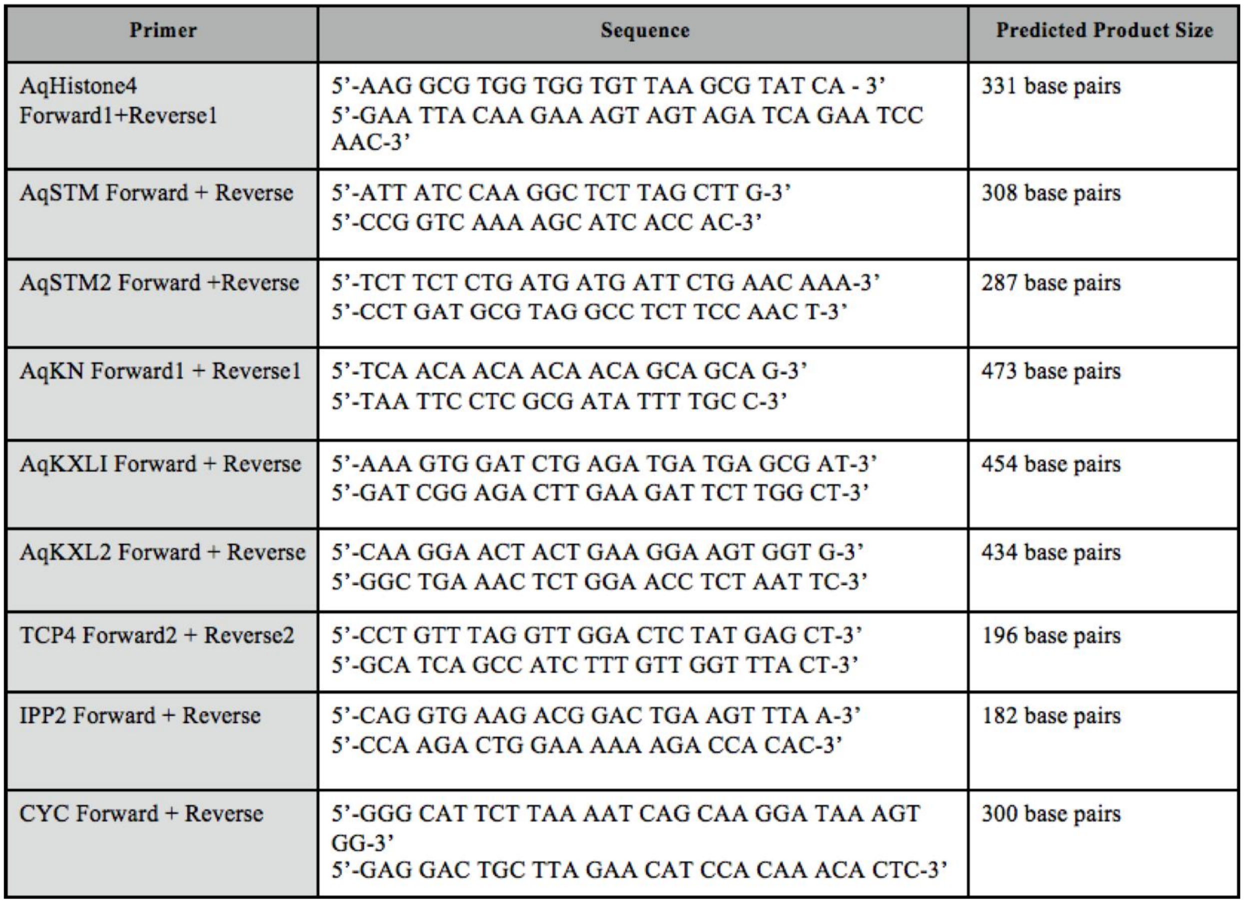
All floral expression studies were performed using Aquilegia coerulea L. "Origami". Plants were grown in the greenhouses of the Dept. of Organismic and Evolutionary Biology, Harvard University.

RNA extraction and cDNA synthesis

RNA was extracted from petals using the Invitrogen Concert prep protocol for small scale extractions (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized following the Invitrogen SuperScript III First-Strand Synthesis protocol (Invitrogen, Carlsbad, CA, USA).

RT-PCR

For each gene of interest, primers specific to that gene were mixed with distilled H20, Taq polymerase, cDNA, and 10x PCR buffer. The mix and cDNA was amplified for 25 cycles at 55 degrees Celsius. Amplification of the housekeeping control gene ISOPENTYL PYROPHOSPHATE:DIMETHYLALLYL PYROPHOSPHATE ISOMERASE2 (AqIPP2) was used as a positive control (Figure 4). See Table 1 for sequences and expected product size from all PCR primers.



Primer	Sequence	Predicted Product Size
AqHistone4 Forward1+Reverse1	5'-AAG GCG TGG TGG TGT TAA GCG TAT CA - 3' 5'-GAA TTA CAA GAA AGT AGT AGA TCA GAA TCC AAC-3'	331 base pairs
AqSTM Forward + Reverse	5'-ATT ATC CAA GGC TCT TAG CTT G-3' 5'-CCG GTC AAA AGC ATC ACC AC-3'	308 base pairs
AqSTM2 Forward +Reverse	5'-TCT TCT CTG ATG ATG ATT CTG AAC AAA-3' 5'-CCT GAT GCG TAG GCC TCT TCC AAC T-3'	287 base pairs
AqKN Forward1 + Reverse1	5'-TCA ACA ACA ACA ACA GCA GCA G-3' 5'-TAA TTC CTC GCG ATA TTT TGC C-3'	473 base pairs
AqKXLI Forward + Reverse	5'-AAA GTG GAT CTG AGA TGA TGA GCG AT-3' 5'-GAT CGG AGA CTT GAA GAT TCT TGG CT-3'	454 base pairs
AqKXL2 Forward + Reverse	5'-CAA GGA ACT ACT GAA GGA AGT GGT G-3' 5'-GGC TGA AAC TCT GGA ACC TCT AAT TC-3'	434 base pairs
TCP4 Forward2 + Reverse2	5'-CCT GTT TAG GTT GGA CTC TAT GAG CT-3' 5'-GCA TCA GCC ATC TTT GTT GGT TTA CT-3'	196 base pairs
IPP2 Forward + Reverse	5'-CAG GTG AAG ACG GAC TGA AGT TTA A-3' 5'-CCA AGA CTG GAA AAA AGA CCA CAC-3'	182 base pairs
CYC Forward + Reverse	5'-GGG CAT TCT TAA AAT CAG CAA GGA TAA AGT GG-3' 5'-GAG GAC TGC TTA GAA CAT CCA CAA ACA CTC-3'	300 base pairs

Table 1: Primer Sequences and Size. RT-PCR used the listed forward and reverse primers to test the expression levels of each respective KNOX gene in Aquilegia petal spurs. Their full primer names, sequences, and predicted product size are shown above. IPP2 is the positive control of the experiment.

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