Allelic promoter variation causes a switch in reproductive tissue expression in an *Arabidopsis* exonuclease gene

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Keywords: Apomixis, APOLLO, *Boechera*, promoter-swap, 5'UTR, reproduction

Abstract

Among candidate genes underlying the control components of apomixis, APOLLO is known for its strong linkage to apomeiosis in the genus *Boechera*. The gene has (Apo-alleles), which are characterized by a set of linked apomixis-specific polymorphisms, and (Sex-alleles). All apomictic *Boechera* genotypes being heterozygous for the Apo/Sex-alleles, while all sexual genotypes are homozygous for Sex-alleles. In this study native and synthetic APOLLO promoters were made and their functions were characterized by detecting the expression level of the β-glucuronidase (GUS) gene in *Arabidopsis* and an apomictic *Boechera*. The data show that there are differences in GUS activity between Apo- vs. Sex-alleles, though no GUS activity was observed in pre-meiotic ovules of Apo-allele transformants. This may mean that the 1 kb and 2 kb regions do not carry all needed regulatory elements and hence a longer promoter region can be tested for future study. Comparing various flower developmental stages in transgenic lines containing the different constructs with the 2 kb native transgenic lines revealed that changes to the APOLLO promoter causes shifts in tissue- and developmental-stage specificity. In conclusion, the 20 bp apomixis-specific polymorphism along with other sequences of the 5'UTR gives rise to ovule-specific GUS activity only when adjacent to 2 kb of the Sex-allele.

Introduction

In contrast to sexual reproduction and segregation of traits in his peas, Mendel's research on *Hieracium* illustrated a distinct kind of inheritance in which segregation was absent), and which was later identified as the parthenogenetic development of meiotically-unreduced egg cells (Bicknell et al, 2016; Mittelsten Scheid, 2022). As with *Hieracium*, many plant species

reproduce through apomixis, a natural form of asexual reproduction through which seeds are produced with embryos which are genetic clones of the mother plant (Hojsgaard and Hörandl, 2019; Underwood and Mercier, 2022).

While apomixis can arise via different mechanisms in natural plant populations (Hojsgaard and Hörandl, 2019; Underwood and Mercier, 2022), it fundamentally differs from sexual seed formation by the production of the meiotically-unreduced egg-cell (apomeiosis) which develops into an embryo without fertilization (parthenogenesis) in addition to endosperm development with or without fertilization of the central cell (Hojsgaard and Hörandl, 2019). Many apomicts are furthermore facultative (i.e., producing both sexually- and apomictically-derived embryos), and hence there is interplay between the reproductive pathways (Koltunow et al., 2011; Bicknell et al., 2003; Fehrer et al., 2007; Aliyu et al., 2010). Apomicts are often interspecific hybrids, which has led to the hypothesis that deregulation of genes underlying sexual seed formation underlies the genesis of apomixis (Carman, 1997). Furthermore, many apomictic plants and parthenogenetic animals are also polyploid, with implications for changes in gene expression and mutational load tolerance (among other traits) as being favorable for apomixis (Hojsgaard, 2018).

Apomixis has received much attention because of its potential ability to freeze complex hybrid genotypes (Tucker and Koltunow 2009). It does not exist in crop plants, and the introduction of apomixis from wild apomictic relatives into crops (Underwood et al., 2022), or via *de novo* induction would be highly disruptive to modern agriculture (Spillane et al., 2004; Underwood et al., 2022). Recent progress in inducing apomixis properties in lettuce and rice demonstrates the importance of research in this field to establish new tools to improve global food security (Underwood et al., 2022; Vernet et al., 2022).

The genus *Boechera* Á. Löve & D. Löve (Boecher's rock cress; Brassicaceae)(Dobeš et al., 2006) is an ideal model system to study the evolution of apomixis, a major reason being that sexual and apomict plants can be compared genetically at the same ploidy level (Corral et al., 2013). The genus is characterized by diploid sexuals (basic chromosome number $x = 7$; $2n = 2x = 14$), as well as diploid ($2n = 2x = 14$ or 15) and triploid ($2n = 3x = 21$) apomicts (Kantama et al., 2007; Alexander et al., 2013; Lovell et al., 2013). A highly reticulate pattern of genetic variation on inter- and intraspecific levels is reflective of numerous diploid and triploid

apomictic lineages in geographically and genetically distinct populations (Rushworth et al., 2011; Rushworth et al., 2018; Kiefer et al., 2009), whereby rare haploid apomictic pollen can spread of apomixis factors to establishment new diploid apomictic lineages (Mau et al., 2021).

The establishment of apomixis-like reproduction in plants, using multiple gene mutants (d'Erfurth et al., 2009), or through genetic constructs based upon candidate loci identified in different wild apomictic species (Underwood and Mercier 2022; Hojsgaard and Hörandl 2019), together demonstrate that multiple functional pathways are available to achieve asexuality. From a functional evolutionary perspective, deregulation of genes underlying sexual seed formation likely involves multiple loci which are simultaneously co-opted to induce apomixis, with changes to tissue-specificity and timing of gene expression leading to the switch from sexual to apomictic seed production in natural populations (Schmidt, 2020).

Here, we demonstrate that sex- vs. apomixis-specific polymorphisms in the 5'UTR of the apomixis candidate APOLLO gene (Corral et al., 2013; Martin et al., 2015) can modify reproductive tissue-specific expression in its orthologue in Arabidopsis. UTR-control of gene expression, has been demonstrated to be significant in other plant species (Srivastava et al., 2018) as well as humans (Babu et al. 2021). For example, a novel motif in the 5'UTR of Big Root Biomass (BRB) gene modulates root biomass in Sesame, whereby this motif in single and duplicated copies was responsible for high and low root biomass in two different accessions, respectively (Dossa et al., 2021). Global spatial analysis of Arabidopsis natural variants revealed 5′UTR splicing of LATE ELONGATED HYPOCOTYL (LHY) in responses to temperature (James et al., 2018). In addition, blue light exposure in Arabidopsis triggers variation in 5'UTR length resulting in differential transcription start site (TSSs) usage which in turn influences the post-transcriptional regulatory processes (D.Georgy, 2018; Yukio et al., 2018).

In *Boechera* APOLLO is part of the DEDD 3′→5′ exonuclease superfamily (Corral et al., 2013), and the expression of its orthologue in *A. thaliana* (AT1G74390), has been reported in flowering stages, mature plant embryos, petal differentiation and expansion stages, plant embryo bilateral stages, plant embryo cotyledonary stages and plant embryo globular stages (https://www.arabidopsis.org). Sexual *Boechera* are homozygous for an APOLLO Sex-allele, while apomicts have both Apo- and Sex-alleles, which differ in sequence composition and structure (Corral et al., 2013). In *Boechera*, Apo-alleles of APOLLO are highly expressed in

developing apomictic ovules during the megaspore mother cell stage, while Sex-alleles are not expressed in apomictic ovules (Corral et al., 2013). No APOLLO expression occurs in the ovules of sexual plants (Corral et al., 2013). Ovule-specific expression of APOLLO in apomictic *Boechera* is hypothesized to be associated with differences in the 5'UTR region which differs between Apo- and Sex-alleles, whereby the Apo-allele 5'UTR is truncated and additionally contains an internal 20 bp insertion in the Apo-allele 5'UTR in comparison to the Sex-allele (Corral et al., 2013). Here, we have employed a promoter-swap experiment between Apo- and Sex-allele specific sequences to investigate the influence of the apomixis-specific polymorphisms on the gene expression of this exonuclease in Arabidopsis.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (Columbia-0 ecotype) seeds were obtained from the Arabidopsis Biological Resource Centre (ABRC). Surface sterilised seeds were sown on MS plates containing ½ MS (Murashige & Skoog) medium (pH adjusted to 5.7 with 1M NaOH) with 0.8% phytoagar, stratified at 4°C for 48 h and then transferred to a phytochamber at 20°C with a light cycle of 16h light (white fluorescent bulbs, with a light intensity of 70 µmol m^{-2} s^{-1}) and 8h dark. Two-week old Arabidopsis seedlings were transferred to pots containing Sunshine mix 8 and grown in the same conditions.

Construct generation and plant transformation

Native and promoter swap constructs were created using various combinations of Sex- versus Apo-specific polymorphisms identified in Corral et al. (2013; Supplemental figure 1). All primers used are described in Supplementary table 2 and 3. All verified construct sequences have been submitted to Gene bank (Supplemental table 5).

To build native *Boechera APOLLO* putative promoter constructs (C1 and C2), approximately 2kb upstream of start codon, including 5'UTR, was amplified from *B. stricta* (ES 718; sexual) and *B. divaricarpa* (ES 517; apomictic) genomic DNA using CloneAmp (Thermo Fischer Scientific) DNA polymerase. Specific primers were designed based on BAC Apo- and Sex-allele sequence libraries (Corral et al. 2013). Synthetic promoter splice variants (C3 to C7) were constructed using a modified "gene Splicing by Overlap Extension" method (SOE) (Horton et

al., 1989). Briefly, SOE consists of two rounds of PCR. In PCR round one (PCR1), promoter region and 5′UTR fragments were amplified separately, with complimentary overhangs incorporated into the reverse and forward primers, respectively. In PCR round two (PCR2), an approximately equimolar ratio of a pair of purified PCR1 products were mixed, and used as a template to amplify the fully spliced sequences, using flanking primers. For C3, C4 and C5, modifications to the 5′UTR indel region were created by modifying the respective primer sequences. Resultant PCR products were purified and cloned into a pENTR™/D-TOPO™ entry vector via directional TOPO® cloning, and incorporated into the pBGWFS7 GFP:GUS reporter vector (Nakagawa et al., 2007) using Gateway™ LR Clonase™.

Binary vectors were transformed into the *Agrobacterium tumefaciens* strain GV3010 by electroporation, and eight-week old wild-type *Arabidopsis* Col-0 plants were transformed by floral dip (Clough and Bent, 1998). Transformed seeds were selected based on the method by Harrison et al. (2006). Briefly, bulk seed were plated to MS plates supplemented with 50 µg/ml ammonium glufosinate, and stratified for 48 h (4°C, dark). Plates were transferred to a phytochamber for 4-6 h at 22°C in continuous white light (120 µmol m^{-2} s^{-1}) to stimulate germination, then incubated in the dark for 2 d at 22 °C. Glufosinate-resistant seedlings were identifiable after a further 48 h at 22ºC in white light (120 µmol m^{-2} s^{-1}). Two-week old glufosinate-resistance seedlings were then transformed to pots containing Sunshine mix 8, and presence of the transgene was confirmed by PCR of genomic DNA.

Histochemical assay of GUS activity

Tissue-specific promoter activity for all constructs were was assessed by β-glucouronidase (GUS) activity; seedlings and flowers from at least 10 different transformation lines were examined per construct. GUS-staining was performed following the protocol of Jefferson (1987). Briefly, two-week old seedling and/or flowers from 32-day old plants were immersed in ice-cold 90% acetone for 20 min, rinsed in water to remove acetone, then washed once with 50 mM sodium phosphate (pH 7.0). Fixed tissues were then incubated in GUS staining solution [0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, 0.1 M $K_4Fe(CN)_6$, 0.1 M $K_3Fe(CN)_6$, 0.5 M EDTA disodium salt (pH 8.0), 0.1% Triton-X and 2 mM X-GLUC in H_2O]. After a vacuum infiltration treatment of 15 min, the immersed tissues were incubated in 37°C for 48 h.

Tissues were cleared following the protocol of Malamy and Benfey (1997). Samples were mounted in 50% glycerol on glass microscope slides. Three plants were used in each treatment. *A. thaliana* var. Columbia that was not exposed to the clearing material was used as a control. Promoter activity in the leaves and flowers of *A. thaliana* was measured by a GUS histochemical assay at two- and five-days post-treatment in a minimum of three flowers per developmental stage (Smyth et al., 1990; Rojek et al., 2018). Images were obtained under a Zeiss Lumar.V12 Stereoscope and Olympus BX61 microscope and analyzed using Image J (https://rsb.info.nih.gov/ij/). To compare GUS positive areas, the linear region of interest (ROI) method was used, whereby a straight line is drawn through cell images to depict the main intensity of the color (Béziat et al., 2017). The original data was normalized by Min-Max method and analyzed using a two-way analysis of variance (two-way ANOVA). Finally, statistical differences were determined by Tuckey test.

Results

(a) A 2kb of native *Boechera* Apo promoter is expressed in the vascular tissues of *Arabidopsis*

Positive GUS activity for root, shoots, and leaves of two-week old seedlings for lines transgenic for the construct pC1 carrying 2 kb native Apo promoter was observed in 33 lines of two-week old seedlings, each with 4 replicates (Figure 1). From this pattern (Figure 1a), three of the 33 lines were negative only for roots, shoots, or leaves, whereas 5 of 33 lines were negative only for roots. In contrast, no GUS activity was observed for 31 lines of two-week old seedlings transgenic for the construct pC2, carrying 2 kb Sex native promoter (Figure 1b). There was some positive GUS activity in leaves of six-week old *Arabidopsis* transgenic line for pC1 (Figure 1c).

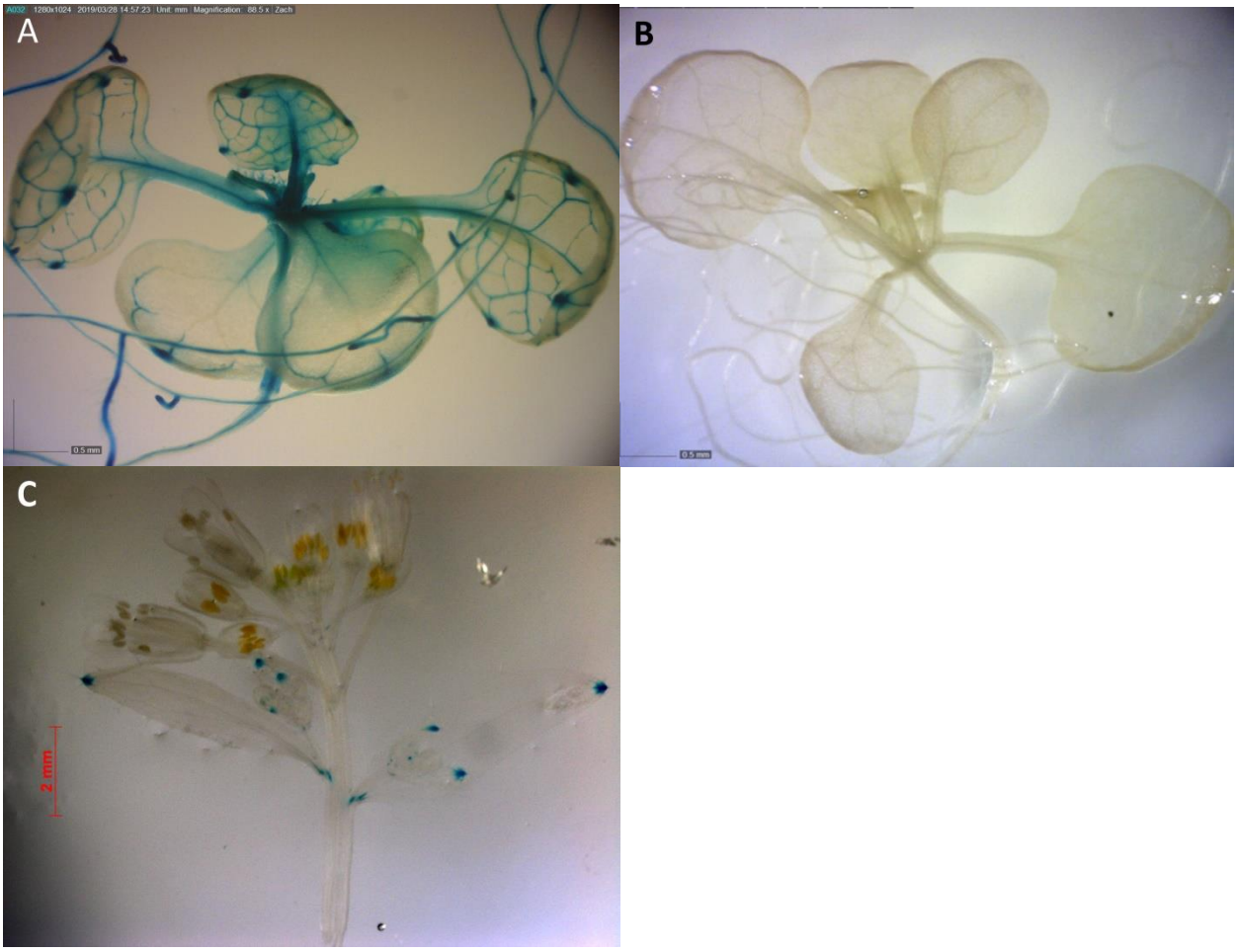


FIGURE 1

Expression patterns of *Boechera* native promoter GUS constructs in *Arabidopsis*. (A) GUS distribution of 2 kb native pApo promoter (pC1) in two-week old plant. (B) No GUS activity observed in 2 kb native pSex promoter (pC2), (C) positive GUS activity in leaf of six-week old *Arabidopsis* transgenic for pC1. Scale bars are labelled.

No other consistent GUS activity was detected in any tissues over a six-week growth period in *Arabidopsis* from 3 transgenic lines for 2 kb native *Boechera* construct pC1 (Figure 1), or for 10 transgenic lines of six-week old *Arabidopsis* for the 2 kb native *Boechera* pC2 construct .

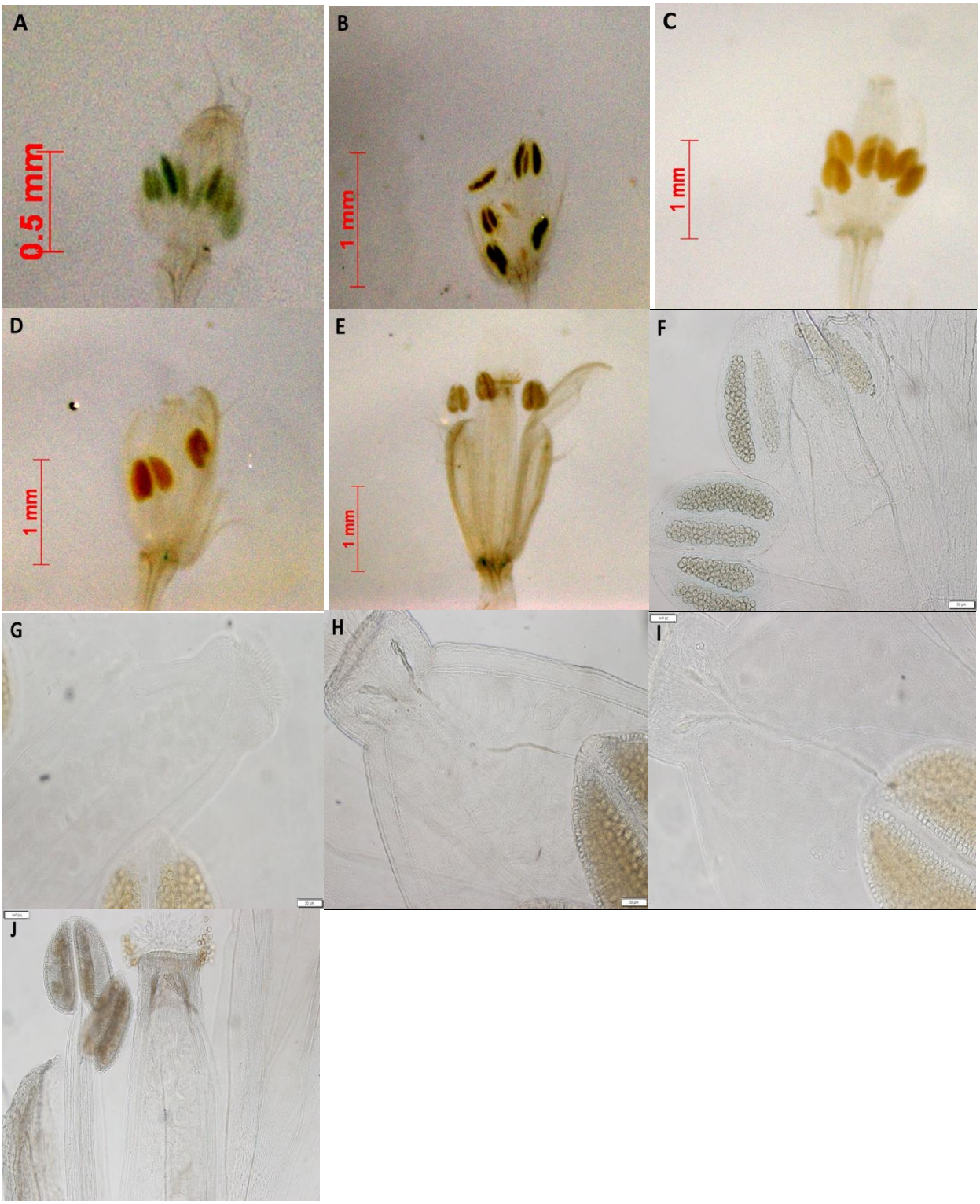


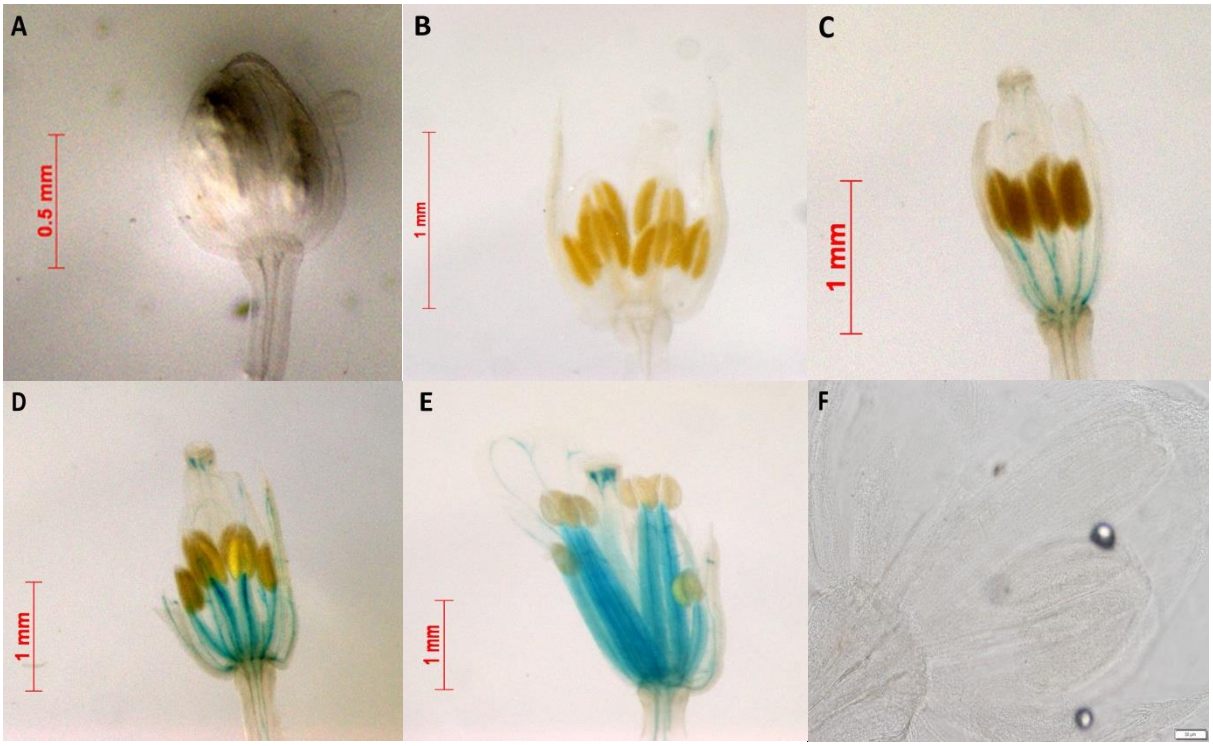
FIGURE 2

Expression patterns of 2kb native Apo promoter GUS constructs in *Arabidopsis* flowers. GUS distribution of pC1 in different developmental stages of mature flowers. (A) 0.5 mm flower with GUS activity (arrow) in anthers Scale bar=0.5 mm. (B) 1.0 mm flower with GUS activity in anthers. (C)1.5 mm flower. (D) 2.0 mm flower. (E) 3.0 mm flower. (b-d) Scale bar=1.0 mm. (F) 0.7 mm flower with minimal GUS activity in

anther wall layer. (G) 1.0 mm flower. (H) 1.5 mm flower. (I) 2.0 mm flower. (J) 3.0 mm flower. For (F-J) Scale bar=50 µm (Olympus BX61 microscope), Scale bar=0.5 mm.

(b) Removal of a 20 bp Apo-specific insertion from the native 2kb Apo-allele induces reproductive tissue-specificity of expression

Construct pC3 carries the 2 kb native pApo promoter, the 5'UTR of Apo-allele, without the 20 bp Apo- insertion (Supplemental figure 1), and GUS activity was measured in 8 transgenic lines each of which having five different developmental stages (total of 40 flower buds). These developmental stages are based on pistil and stigma maturity according to Smyth et al. (1990) and Christensen et al. (1997) that we use throughout this work. No GUS activity was observed for both stigma and ovule in the pre-meiotic (0.7 mm) and post meiotic (1.0 mm) stages, while positive GUS activity was observed for stages 3, 4, and 5 in the stigma and filament with an increasing activity toward the final stages (Figure 3c, d, e, $P \leq 0.05$, Tuckey test; Supplemental figure 2). Dispersed GUS activity in petals was excluded because they did not show a constant pattern for all samples



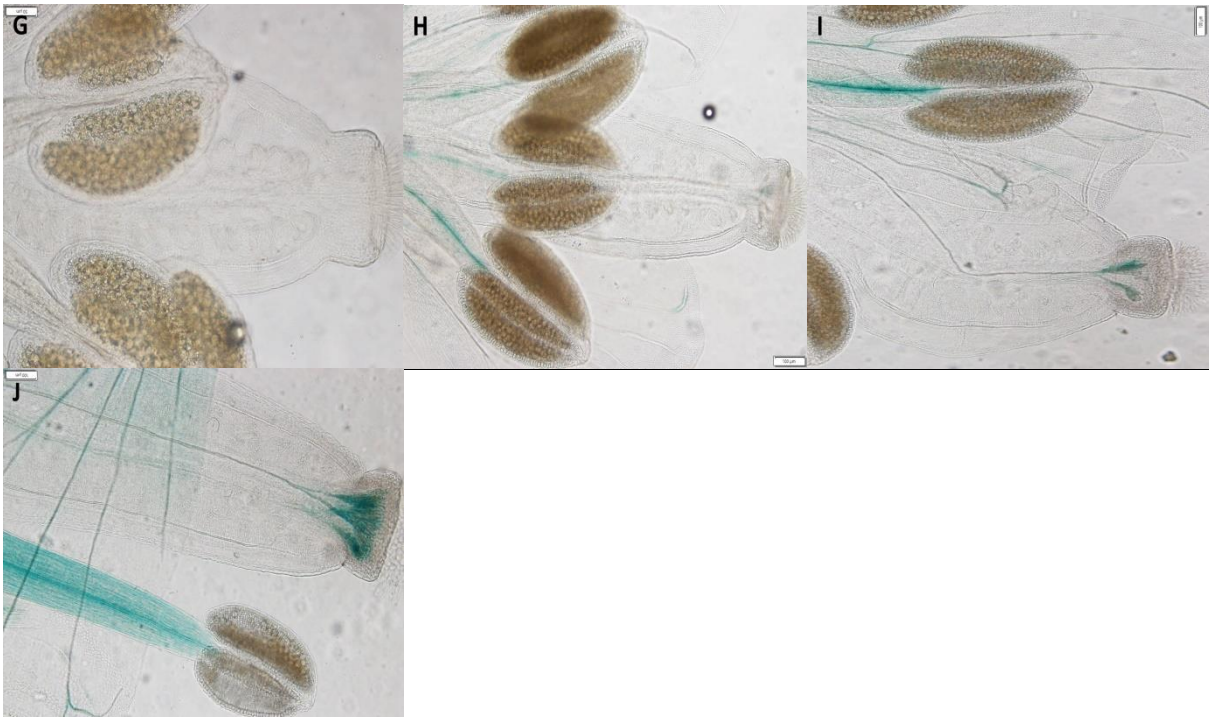


FIGURE 3
Expression patterns of synthetic promoter construct (pC3: GUS) in *Arabidopsis* flowers. Construct pC3 promoter in different developmental stages of flowers. (A) 0.7 mm flower Scale bar=0.5mm. (B) 1.0 mm flower with GUS activity on petals. (C) 1.5 mm flower with GUS activity on filament. (D) 2.0 mm flower with GUS activity on filament and stigma. (E) 3.0 mm flower with GUS activity on filament and stigma. (F) 0.7 mm. (G) 1.0 mm flower. (H) 1.5 mm flower. (I) 2.0 mm flower. (J) 3.0 mm flower, (F-J) Scale bar=100 µm (Olympus BX61 microscope). (A-E) Scale bar=1.0 mm (Zeiss Lumar.V12 Stereoscope).

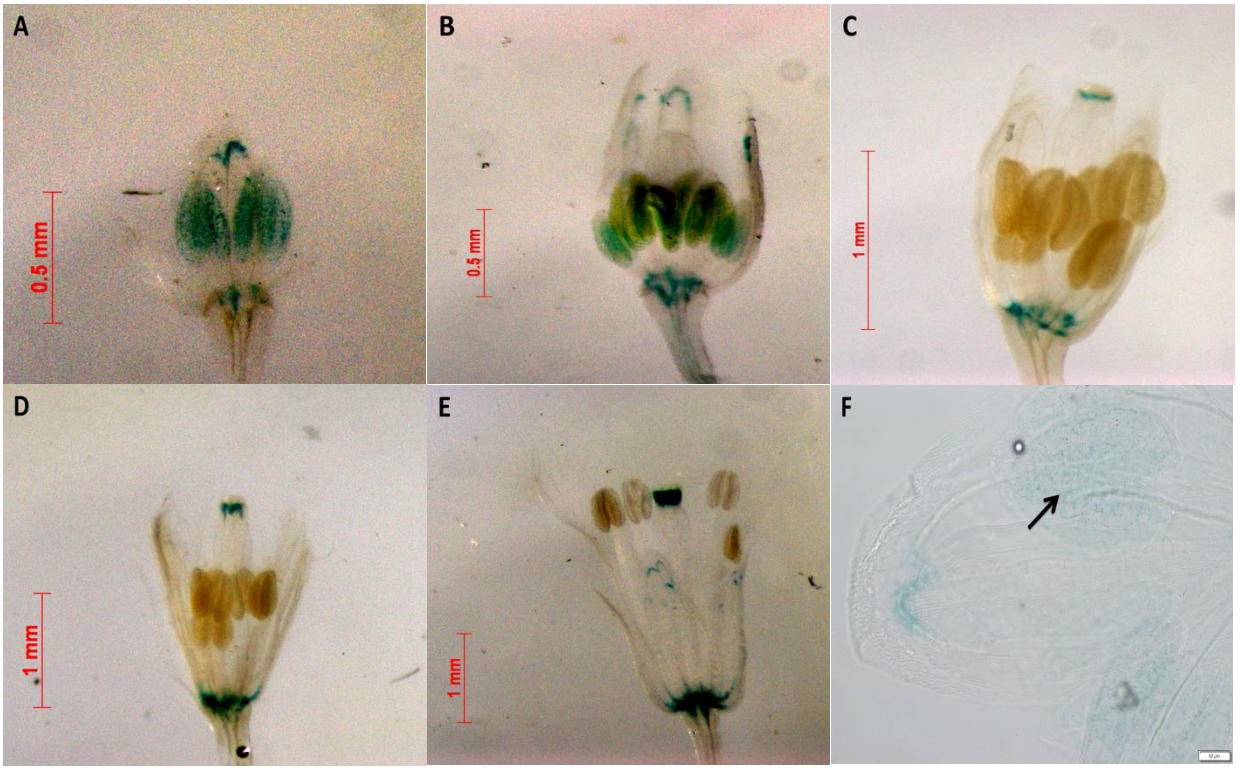
(c) A 20 bp Apo-specific insertion does not induce expression in the 2kb native *Boechera* sex-allele

Construct pC4 carried the 2 kb native pSex promoter, the 5'UTR of the Sex-allele, with the 20 bp Apo-insertion. The GUS activity for 7 transgenic pC4 lines, each of which had five different developmental stages (total of 35 flower buds) was measured, and no GUS activity was detected (Supplemental figure 3).

(d) Randomizing the 20 bp Apo-specific insertion but maintaining size induces anther and stigma expression of the native Apo-promoter

Construct pC5 was composed of the 2 kb native pApo promoter plus the 5'UTR of the Apo-allele, but a 20 bp random insertion was substituted for the apomixis-specific 20 bp insertion (Supplemental figure 1). GUS activity was observed in the anthers and receptacle for the pre-

meiotic stage, as well as some anther-specific GUS activity for stages 2 (Figure 4; Supplemental figure 4). Stigma-specific GUS activity increased over stages 3, 4, and 5 (Figure 4b, c, d, and e). GUS activity for the flower receptacle, and petals were excluded for quantification because they did not show a constant pattern for all samples.



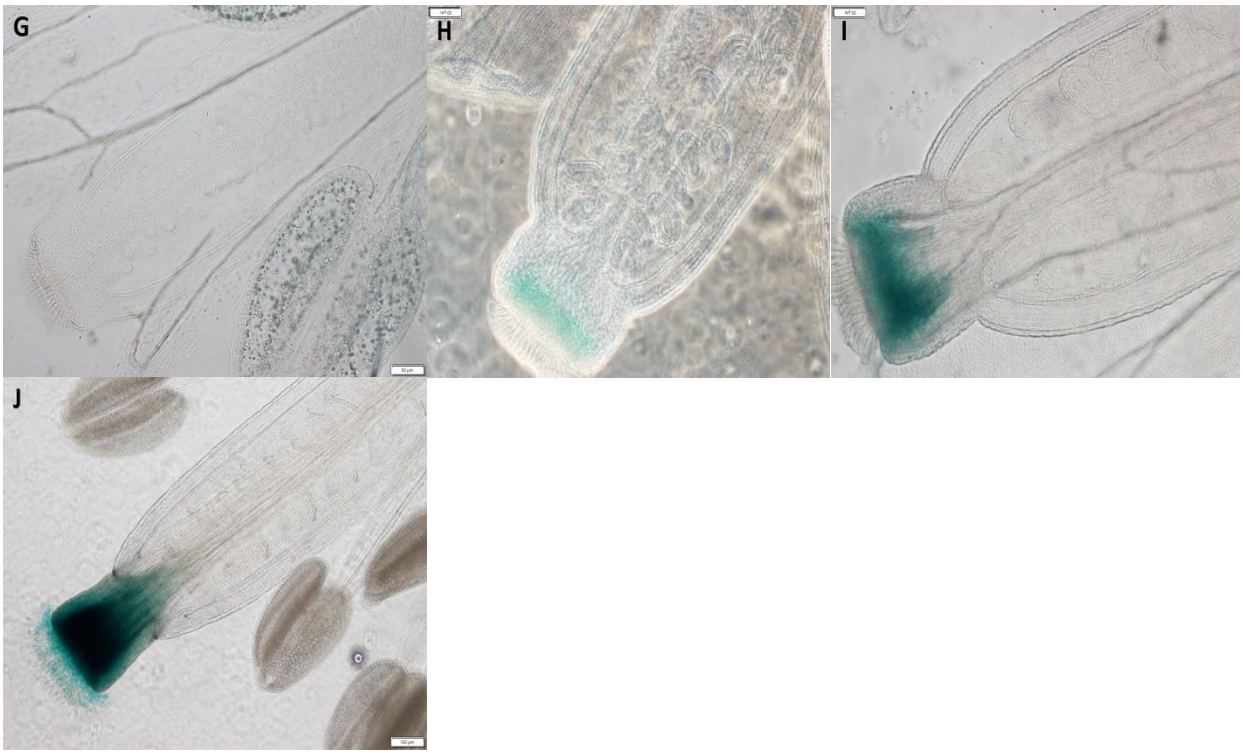
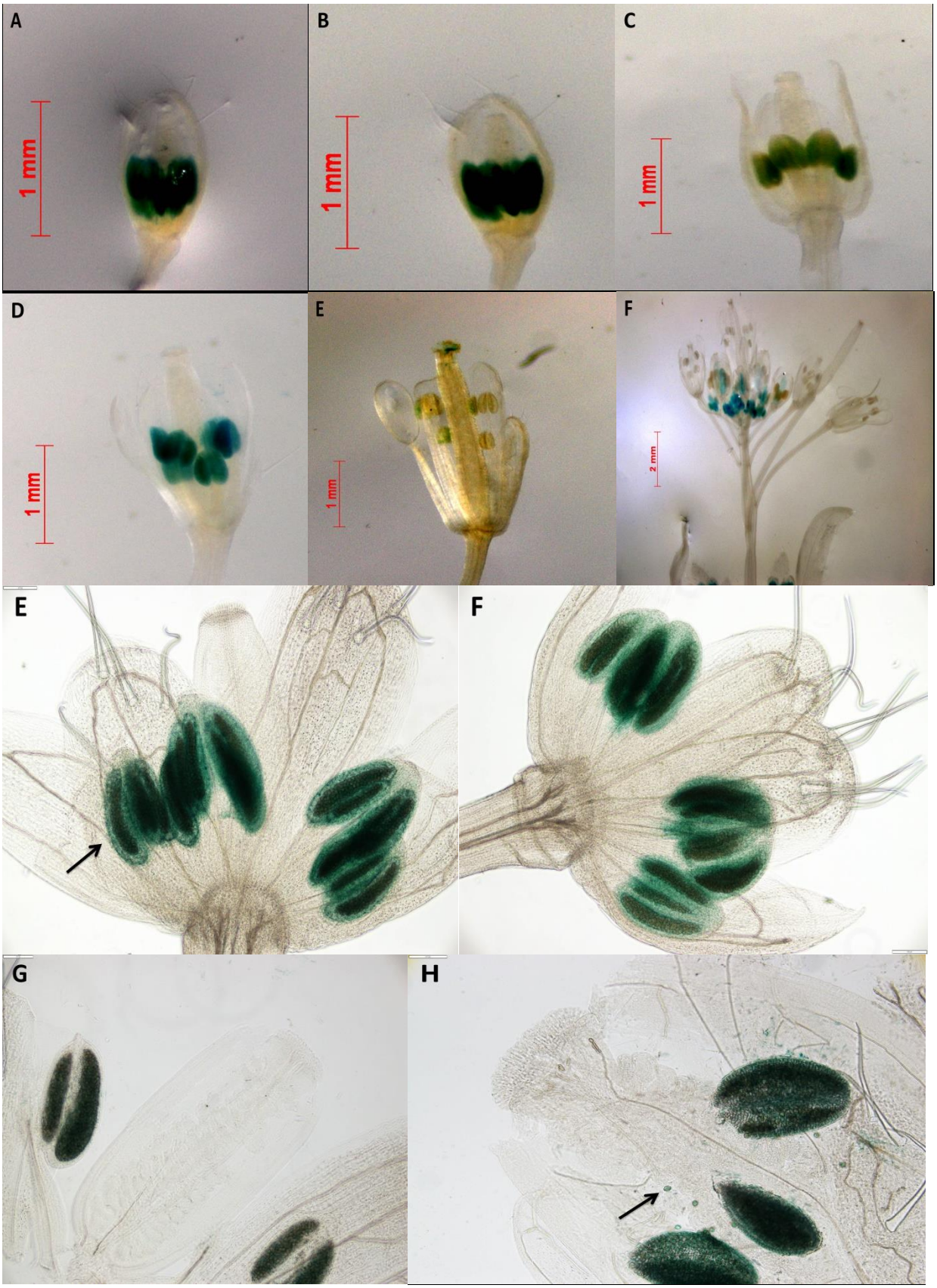


FIGURE 4
Expression patterns of spliced promoter construct (pC5: GUS) in *Arabidopsis* flowers. Construct pC5 promoters in different developmental stages of flowers. (A) 0.7 mm flower, Scale bar=0.5 mm. (B) 1.0 mm flower with GUS activity in anther and receptacle. (C) 1.5 mm flower with GUS activity in stigma and receptacle. (D) 2.0 mm flower with GUS activity in stigma and receptacle. (E) 3.0 mm flower with GUS activity in stigma and receptacle. (A-E) Scale bar=1.0 mm (Zeiss Lumar.V12 Stereoscope). (F) 0.7 mm flower with GUS activity in anther and receptacle. (G) 1.0 mm flower with GUS activity in anthers. (H) 1.5 mm flower with GUS activity in stigma and receptacle. (I) 2.0 mm flower with GUS activity in stigma and receptacle. (J) 3.0 mm flower with GUS activity in stigma and receptacle. (G-J) Scale bar=50 µm (Olympus BX61 microscope). Pollen-specific GUS expression is shown with black arrow (F)

(e) The Apo-allele promoter with substituted Sex allele 5'- UTR induces exprression in polllen

Construct pC6 carried the 2 kb pApo promoter, with a swapped native Sex-allele 5'UTR of Sex-allele (Supplemental figure 1) and was analyzed in 4 transgenic lines over five different developmental stages (total of 20 flower buds). Anther-specific GUS activity with varying intensity was observed for all developmental stages (Figure 5), with the strongest expression in stage 2 (Figure 5b; $P \leq 0.05$; Tuckey test; Supplemental figure 5). We hypothesize that pollen activity is induced (Figure 5f, h, I, j), as is evidenced by post-dehiscence loss of activity (Figure 5d to e).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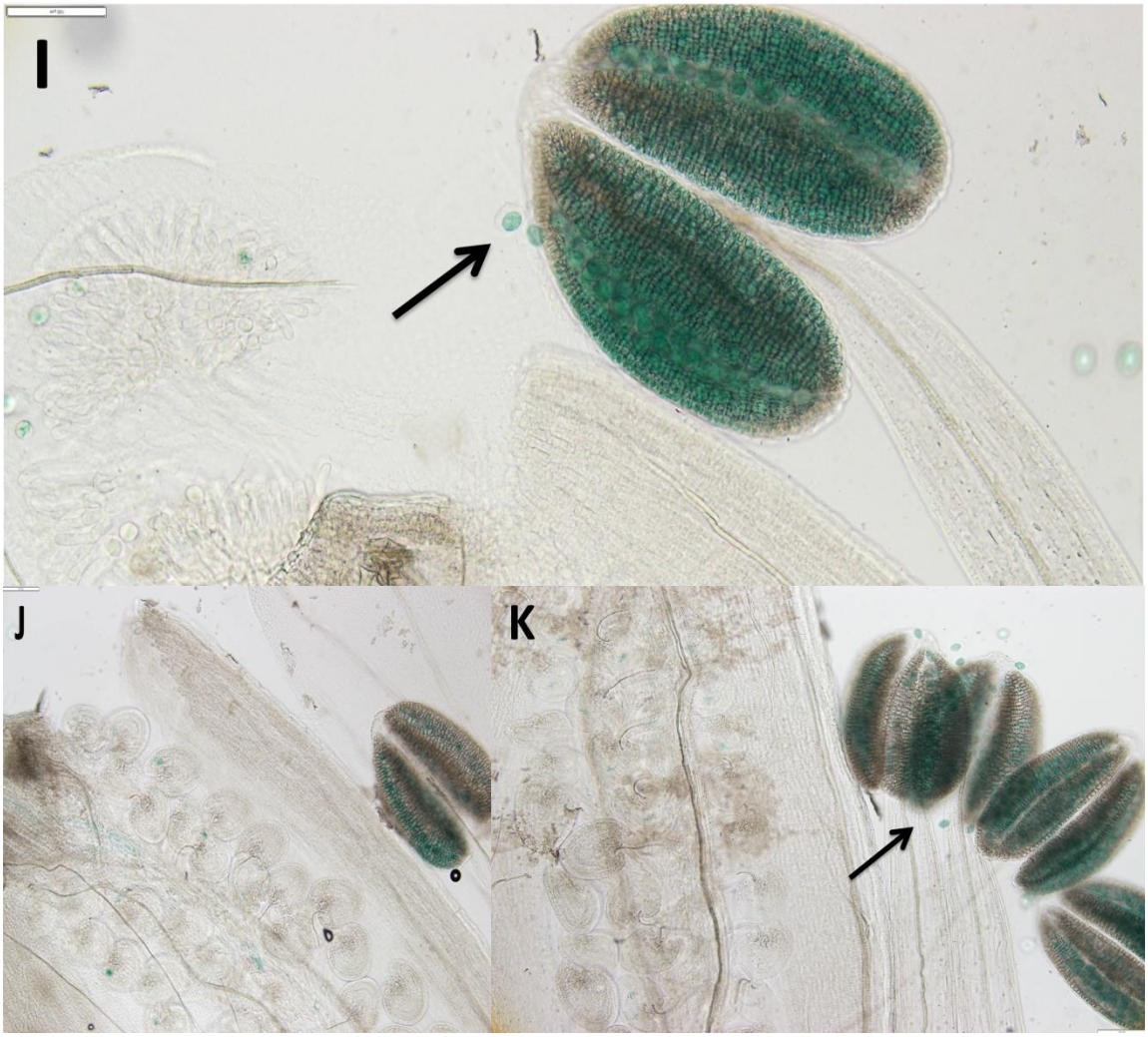
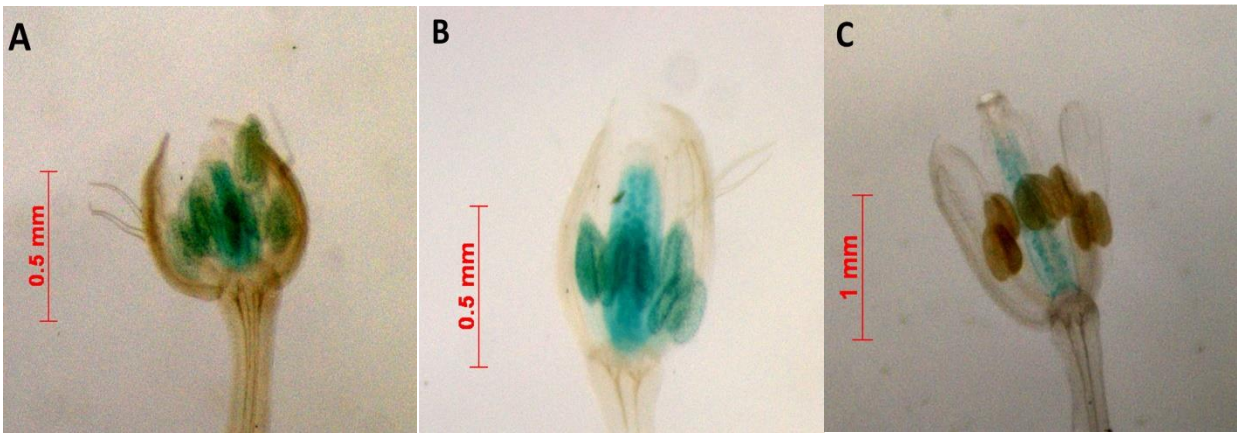


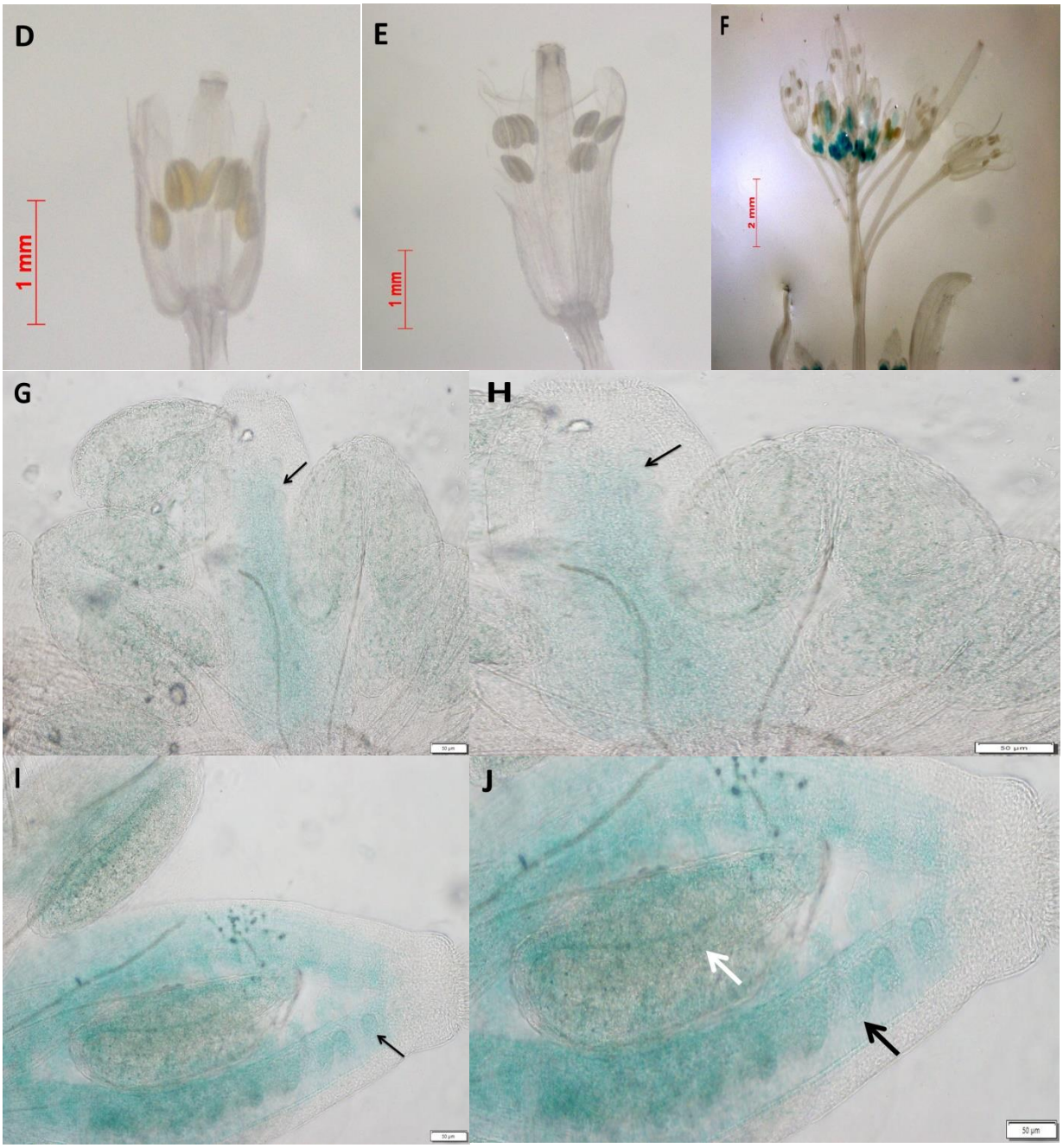
FIGURE 5
Expression patterns of spliced promoter construct (pC6: GUS) in different developmental stages of *Arabidopsis* flowers: (A) 0.7 mm (B) 1.0 mm, (C) 1.7 mm (D) 2.0 mm, (E) 3.0 mm, (A-E) Scale bar=1.0 mm (Zeiss Lumar.V12 Stereoscope), (F, G) 0.7 mm, (H) 1.0 mm, (I) 1.5 mm flower, (J) 2.0 mm, flower, (K,L) 3.0 mm, (F-L) Scale bar=50 µm (Olympus BX61 microscope). Pollen-specific GUS expression demonstrated by black arrows (E, H, I, K).

(f) The Sex-allele promoter with substituted Apo-allele 5' UTR induces a burst of expression in both pollen and ovules in pre-zygote stages, followed by decreasing expression in zygotes

Construct pC7 carried the 2 kb pSex promoter with a swapped Apo-allele 5'UTR (Supplemental Figure 1), and was analyzed in 6 transgenic lines over five developmental stages (total of 30 flower buds). GUS activity with a varied intensity was observed in the style, ovules and anthers for the first two developmental stages (Figure 6a, b), while only ovule expression occurred in

stage 3 (Figures 6c, g, h). In stage 4 (Figure 6d, f), expression in both tissues ceased. Embryo expression began in stage one (Figure 6) and lasted for three stages ($P \leq 0.05$; Tuckey test; Supplemental figure 6)(Tables 1 and 2).

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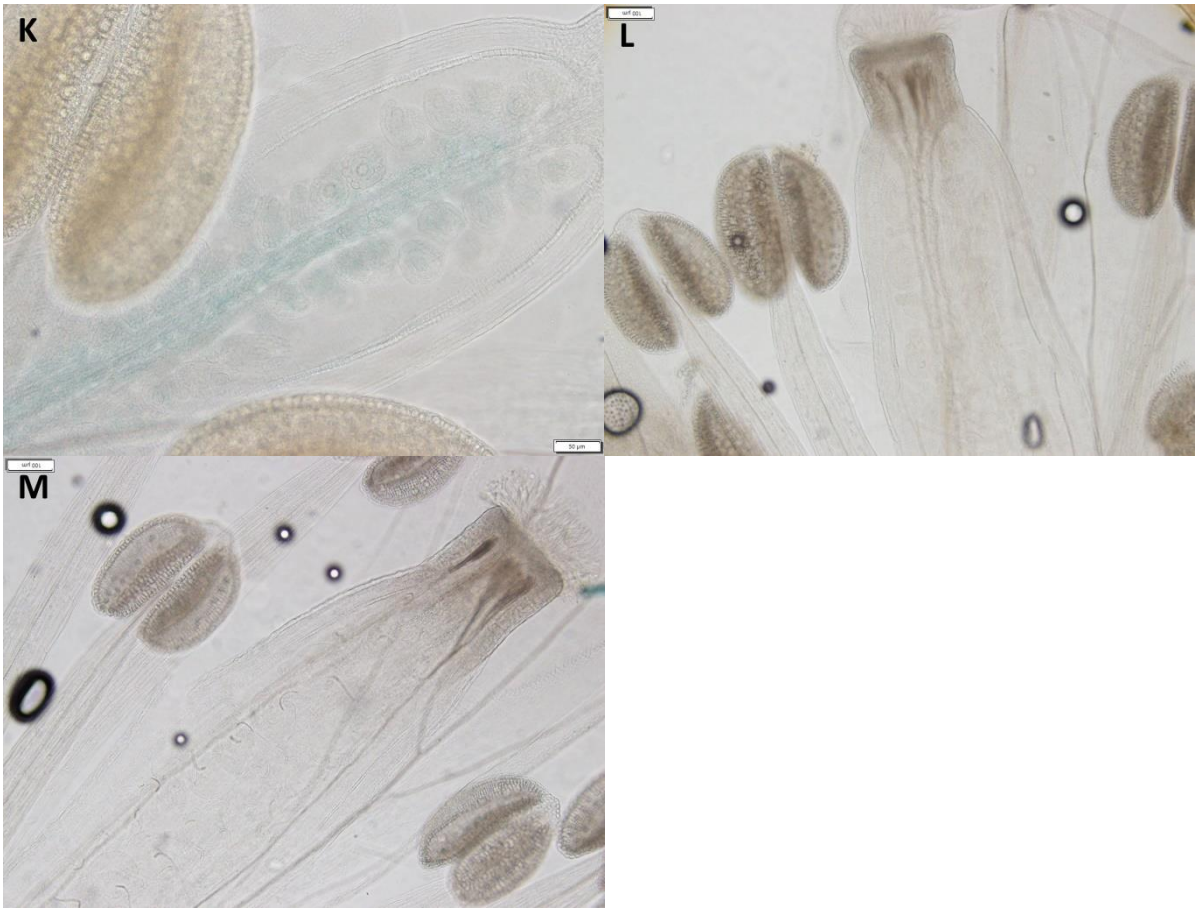

FIGURE 6

Expression patterns of spliced promoter construct (pC7: GUS) in different developmental stages of *Arabidopsis* flowers. (A) 0.7 mm flower with GUS activity in anther and style. (B) 1.0 mm flower with GUS activity in anther, style and ovules. (C) 1.5 mm flower with GUS activity in ovules. (D) 2.0 mm flower, (E) 3.0 mm flower, (A-E) Scale bar=0.5 and 1.0 mm (Zeiss Lumar.V12 Stereoscope). (F) *Arabidopsis* (G, H) 0.6 mm flower with GUS activity in anther and style. (I, J) 1.0 mm flower with GUS activity in anther and ovules. (K) 1.5 mm flower with GUS activity in anther and ovules. (L) 2.0 mm flower. (M) 3.0 mm flower. (F-J) Scale bar=100 µm (Olympus BX61 microscope). Ovule-specific GUS expression demonstrated by black arrows (G, H, I, J), and Anther- specific GUS activity is shown by white arrow (J).

TABLE 2 The results of *Arabidopsis* transgenic for 2 kb native promoter and *Arabidopsis* transgenic for five different synthetic constructs.

Construct	Promoter	5'UTR	20 bp Apo-insertion	GUS leaf	GUS flower
pC1	Apomictic	Apomictic	positive	+	-
pC2	Sexual	Sexual	Negative	-	-
pC3	Apomictic	Apomictic	Negative	+	Filament/Stigma
pC4	Sexual	Sexual	Positive	-	-
pC5	Apomictic	Apomictic	Random insertion	+	Anther/Stigma
pC6	Apomictic	Sexual	Negative	+	Anther
pC7	Sexual	Apomictic	Positive	-	Style/Ovule/Anther

TABLE 3 Comparing the GUS activity in different floral tissues of all constructs based on five different developmental stages (Smyth et al., 1990). Only stage 1 represents the pre-meiotic stage of flower development and the other stages represent post-meiotic stages.

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
pC1	-	-	-	-	-
pC2	-	-	-	-	-
pC3	-	-	Filament/Stigma	Filament/Stigma	Filament/Stigma
pC4	-	-	-	-	-
pC5	Anther/Stigma	Anther/ Stigma	Anther/Stigma	Stigma	Stigma
pC6	Pollen	Pollen	Pollen	Pollen	Pollen
pC7	Style/Ovule/Anther	Style/Ovule/Anther	Ovule	-	-

Discussion

A comparison of gene expression in the micro-dissected ovules of apomictic versus sexual *Boechera* led to the identification of the APOLLO gene. Heterozygous nature and presence of apomixis-specific polymorphisms were proposed to underlay gene expression differences between pre-meiotic ovules of apomictic versus sexual *Boechera* (Corral et al., 2013). Here, a systematic gene expression survey of native and synthetic APOLLO in transformed Arabidopsis was performed to investigate whether there were functional differences between conserved Sex- versus Apo-allele polymorphism. While the native *Boechera* Sex-allele promoter was never expressed in somatic tissues (Fig. 1), promoter swap plants demonstrated a range of expression through development in both male and female reproductive tissues. Furthermore, temporal changes of expression (e.g. expressed in pollen and stigma at the same time; bursts of expression during pre-zygotic stages) are consistent with the action of regulatory factors involved with reproduction in male and female tissues of Arabidopsis.

The native *Boechera* Apo-allele promoter is more expressionally active in Arabidopsis than the Sex-allele promoter

(a) 1 kb native *Boechera* promoters do not drive gene expression in *Arabidopsis*

In the experiment with the 1 kb promoter, no GUS activity was observed for APOLLO Apo- or Sex-allele in transgenic *Arabidopsis* lines. Knowing that 1 kb of the eukaryotic promoter carries a TATA box (Supplemental figure 7 and 8) and/or other sequences needed for attaching RNA

polymerase, which is necessary for transcription initiation, there may be some other elements needed to induce gene expression that are located upstream of the 1 kb region of the APOLLO promoter.

(b) Expression of 2 kb native pApo occurs in somatic tissues

Boechera transgenic lines for the 2 kb native pApo promoter demonstrate expression in the vascular tissues of roots stems and leaves, and expression ceases before the development of reproductive tissues.

(c) No GUS activity observed in apomictic *Boechera* transgenic for 2 kb native Sex-promoter

While there was GUS activity in the stigma for the 2 kb native Apo promoter in the apomictic *Boechera* transformant, none was observed for the 2 kb Sex promoter, and this is consistent with the observations of Corral et al. (2013). The introgression of a sexual allele containing the 20 bp Apo-insertion from a sexual ancestor into other *Boechera* species through hybridization, followed by its fixation due to the lack of recombination (i.e., apomixis) has led to the establishment of the heterozygosity for APOLLO in apomictic *Boechera* (Corral et al., 2013). Thus, the trans-acting effects of TFs on Sex- versus Apo-alleles led to their altered GUS activity in apomicts relative to sexuals (Corral et al., 2013). The architectural differences between the Apo- and Sex-allele may result in either the recruiting of different TFs, or the same TFs with a divergent function.

(d.1) GUS activity for *Arabidopsis* lines transgenic for pC3

Transgenic pC3 lines carry the 2 kb native pApo promoter and the 5'UTR of the Apo-allele without 20 bp Apo-insertion (i.e., the Sex-allele deletion)(Supplemental figure 1). Compared to the transgenic lines for pC1 (2 kb native pApo promoter) where there were no GUS activity, pC3 showed GUS activity in filaments and stigma in earlier and later stages of flower development respectively. Thus, the absence of the 20 bp Apo-insertion led to expression in male tissue, in addition to changing timing of expression. Deletion of the 20 bp Apo-insertion thus likely leads to the loss of the recognition site for regulating interaction elements that control APOLLO expression in specific floral tissues. The shift from filament to stigma in later stages of flower

development also may mean that the localization of GUS activity for pC3 is fertilization-dependent or affected by specific signals from pollen. This hypothesis could be tested by making pC3 transgenic lines in which fertilization is prevented.

(d.2) GUS activity for *Arabidopsis* lines transgenic for pC4

Transgenic pC4 lines carry the 2 kb native pSex promoter, and the 5'UTR of Sex with the 20 bp Apo-insertion (Supplemental figure 1). Knowing that except for the 20 bp Apo-insertion every other part of this construct belongs to Sex-allele, it can be inferred that the APOLLO specific polymorphism itself is not sufficient to produce any GUS activity, or the 2 kb upstream promoter region of the Sex-allele is not enough to drive expression.

(d.3) GUS activity for *Arabidopsis* lines transgenic for pC5

Transgenic pC5 lines carry the 2 kb native pApo promoter, and the 5'UTR of Apo-allele with a random 20 bp insertion (i.e., Apo insertion but different sequence)(Supplemental figure 1). Thus, the insertion of the random 20 bp down-stream from the Apo promoter and 5'UTR of Apo changes the GUS tissue-specificity pattern. This observation is similar to that of pC3, in that the absence of 20 Apo-insertion has led to the loss of recognition sites for Apo interacting factors. The observation of pC5 transgenic lines shows manipulation of the 20 bp insertion led to stigma, receptacle, and anther-specific GUS activity (Figure 4).

(e.4) GUS activity for *Arabidopsis* lines transgenic for pC6

Transgenic pC6 lines carry 2 kb of the Apo promoter and the 5'UTR of the Sex-allele (i.e., with a 20 bp deletion)(Supplemental figure 1). Histochemical GUS staining yielded a prominent signal in anthers which was observed in both pre-meiotic and post-meiotic floral buds (Figure 5). As the native Apo-allele promoter (pC1) drives expression in *Arabidopsis* while the native Sex-allele promoter (pC1) does not, the data here demonstrate that the Apo-promoter upstream of the 5'UTR drives flower-specific expression, while the 5'UTR is responsible for male- or female-specific tissue expression. The observation of this pattern in pre-meiotic and meiotic stages can be interpreted as the association of pC6 with an unknown meiotic function in male tissues.

(d.5) GUS activity for *Arabidopsis* lines transgenic for pC7

Finally, transgenic pC7 lines carry the 2 kb native Sex promoter, 5'UTR of the Apo-allele with the 20 bp Apo-insertion (Supplemental figure 1). This construct shows ovule-, style- and anther-specific GUS activity in earlier stages of flower development (Figure 6). As the native Sex-allele promoter (pC2) showed no activity in *Arabidopsis* flowers, these data demonstrate that the Apo 5'UTR can drive both male- and female-specific expression of APOLLO. This is consistent with the data of Corral et al. (2013), which showed that the Apo-allele 5'UTR of *Boechera* is relatively similar in sequence to *Arabidopsis* and *Brassica* compared to the Sex-allele 5'UTR, and support the hypothesis that the Apo-allele 5'UTR is phylogenetically-older, having originally been derived from sexually-reproducing species.

It is unclear whether the pC7 promoter achieved its novel GUS activity pattern through positive and/or negative gene regulation associated with elements residing in the 2 kb Sex promoter and Apo-allele 5'UTR (with Apo-insertion). Furthermore, this construct is the only one that shows GUS activity in pre-meiotic ovules (Figure 6), which implies the 5'UTR of Apo-allele underlies its regulation.

One explanation for the pC7 construct data is that the substitution of the Apo- with the Sex-promoter has limited access of the regulatory machinery to only the 5'UTR of the Apo-allele (carrying 20 bp Apo-insertion), whereby the 2 kb Sex promoter did not contribute to expression. Thus, it can be implied that the 5'UTR of the Apo-allele is the main player driving ovule-specific GUS activity. Another possible explanation is that the 2 kb Sex promoter contains motifs that actively contributed to the observed GUS activity pattern, although this is not consistent with the negative native Sex promoter activities in *Arabidopsis*.

Despite the importance of the nearby sequences, the existence of distal enhancers cannot be ruled out. Studies on chromosome conformation show that even distal enhancers are commonly in physical proximity with their target genes. In fact, in higher eukaryotic cells, regulatory elements can reside up to hundreds of kilobases away from the genes they control, but can be brought into spatial proximity through chromatin loop formation (Deng and Blobel 2017). Therefore, another comprehensive study by using techniques such as Hi-C (Lieberman-Aiden et al., 2009) could shed light on the importance of distal enhancers on the APOLLO gene expression.

In summary it can be inferred that deletion and randomization of 20 bp Apo-insertion led to changes in tissue-specificity patterns of GUS activity compared to that of native promoters. It can be concluded that the 20 bp Apo-insertion changes the GUS activity from female to male tissues. For instance, the absence of the 20 bp Apo-insertion from the 2 kb native Apo promoter (pC1) which formed construct pC3, produced additional filament-specific GUS activity. The addition of the 20 bp Apo-insertion into the native Sex 5'UTR to create construct pC4 did not change the GUS activity from that of pC2 (i.e., no GUS activity observed for both). Finally, the randomization of the 20 bp in native Apo promoter to create pC5 lead to changes in timing and locality of GUS activity indicating that the Apo-insertion is an important factor in APOLLO Apo-allele regulation.

Constructs pC6 and pC7 had different structures from previous synthetic promoters. pC6, with the Sex-allele 5'UTR combined with the 2 kb of Apo promoter showed a prominent anther-specific GUS activity which started in meiotic stages and shifted to egg-cell specific GUS activity after fertilization. pC7, with the Apo-allele 5'UTR combined with the 2 kb Sex promoter showed prominent ovule, style and anther-specific GUS activity in early stages of flower development. It can be inferred that the original 5'UTR of Sex- or Apo-allele can cause male and female-specific GUS activities respectively, and importantly this happens early on during flower development. In conclusion, the 20 bp Apo-insertion including its surrounding sequences are correlated with female-specific GUS activity. Likewise, the result of another study showed that an auxin-responsiveness in SAUR10 clade genes was affected by cis-element combinations residing in their upstream regions (van Mourik et al., 2017).

The analysis of these synthetic constructs showed that specific parts of Apo-promoter, specifically the 5'UTR and 20 bp Apo-insertion, are associated with expression of APOLLO in female tissues. In this light, the behavior of the APOLLO allele promoters is similar to that of genes in sex-determining regions (SDR) of sex chromosome (Renner and Müller 2021). In sex chromosomes, genes with male function accumulate on the Y chromosome over time, and likewise, genes with the female-biased function will accumulate on the X chromosome (Harkess et al., 2020). The data gained from the study on seven promoter constructs demonstrated that Apo-promoter sequences, specifically the 5'UTR and the 20 bp Apo-insertion drive GUS activities in female tissues (ovule and stigma), while the Sex promoter 5'UTR contributes to

driving expression in male tissues (anther). Considering that apomixis is *per se* a female trait, female-specific expression of genes controlling apomixis is expected and supported by the data presented here.

Another characteristic of sex chromosomes reflected in the APOLLO gene is that the crossing-over is suppressed in the segments of sex chromosomes which contain the SDR genes (Darlington, 1931; Westergaard, 1958). Crossing over is by definition suppressed around the 20 bp Apo-insertion located in the promoter of the APOLLO Apo-allele as a result of apomixis. Thus, it can be implied that APOLLO Apo- and Sex-promoters possibly act similarly to that of sex-linked genes. Based on this model, the Sex promoter contributes to normal meiosis and sexual development, while the Apo-promoter participates in apomeiotic ovule development in *Boechera*, a female-linked characteristic. Another example of sex-linked genes in plants is *NO TRANSMITTING TRACT* (*WIP2/NTT*) in *Arabidopsis*, which encodes a CH2H/C2HC zinc finger transcription factor that is specifically expressed in the transmitting tract and funiculus of ovules, playing a key role in fruit development (Harkess et al., 2020).

(e) Hybridization is associated with the differential gene expression between apomictic versus sexual *Boechera*

The elevated heterozygosity in diploid apomictic *Boechera* can be explained by widespread hybridization associated with the transition from sexuality to asexuality, or it could be a byproduct of apomixis itself. In apomictic individuals the overall heterozygosity is expected to increase over generations due to the lack of recombination and mutation accumulation (Meselson effect; Hojsgaard and Hörandl, 2015). Based on the result of one study on an apomictic *Boechera* population, there was clear evidence for the hybrid origin of the highly heterozygous apomicts (Beck et al., 2012). In addition, mutation accumulation on a genomic level in apomictic *Boechera* has clearly been shown in conserved non-coding sequences (Lovell et al., 2017).

Unlike apomictic *Boechera* the sexual diploid species such as *B. stricta*, are typically less heterozygous and are divergent only at neutral loci (Beck et al., 2012). The interspecific "collision" of different sexual genomes, may produce apomictic *Boechera* with some structural changes in the genome such as homeologous chromosome substitutions and aneuploidy (Kantama et al., 2007). If divergence extends to the regulatory genes, apomixis as a novel

characteristic could result from the novel pattern of gene expressions created by the interaction of divergent transcriptional regulators (J. C. Carman 2001). The data reported in this study show that the variation observed in GUS activity between transgenic lines for pApo native vs. pSex promoters is consistent with the divergent theory of transcriptional regulators and supports the phylogenetic hypothesis proposed in Corral et al. (2013).

The similarity of the APOLLO gene in *Boechera* with that in *A. thaliana* was one of the reasons that transgenic lines of *Arabidopsis* were used for studying synthetic promoters (i.e., monophyletic origin of APOLLO; Corral et al., 2013). The difference reported in GUS activity between *Arabidopsis* and *Boechera* spp. for the 2 kb native pApo construct is likely associated with species-specific regulatory machinery, although the differences in their TF networks is minor, such that *Arabidopsis* can reliably be used for studying APOLLO functionality.

(f) The APOLLO function in *Boechera* probably is associated with cyclins or other meiotic regulators

The variation of the GUS activity patterns observed for native and synthetic constructs may mean that their expression is associated with the cell cycle. For example, in construct pC7 ovule-specific GUS activity was observed in the pre-meiotic stage which decreased over time. The phase-dependency and tissue-specificity of GUS activity reported here may imply that there is a correlation between the function of APOLLO with cyclins or other meiotic regulators. Cyclins are a family of proteins that controls cell cycle progression by activating cyclin-dependent kinase (CDK) enzymes or groups of enzymes required for synthesis (Galderisi et al., 2003). It has been demonstrated that the duplication and evolution of cyclin genes in plants was also accompanied by functional diversification (Bulankova et al., 2013). The data gained from another study which compared gene expression profiles of the Apomictic Initial Cell (AIC) and the Megaspore Mother Cell (MMC) between apomictic *Boechera* and sexual *Arabidopsis* suggests that protein ubiquitinylation and degradation, as well as cell cycle control, may be differentially regulated between the MMC and AIC (Schmidt 2020). The manipulation of cell cycle progression or meiotic genes has also been shown to lead to the formation of unreduced gametophytes (d'Erfurth et al. 2009). Finally, an enrichment in expression of cell cycle related genes was also documented in *Hieracium prealtum* AIC and embryo sacs (Okada et al. 2013).

In addition, diploid heterozygous apomictic individuals could take advantage of complex gene regulation mechanisms arising from bidirectional promoter activity. In practice, bidirectional promoters can produce variable products and relative numbers of transcripts produced via several regulatory steps during the transcription cycle (initiation, elongation, and termination). It was shown that in cases of bidirectional promoters, many of the promoter segments between two bidirectional genes initiate transcription in both directions and contain shared elements that regulate both genes (Trinklein et al. 2004). Bidirectional transcription itself is classified into those affecting the bidirectional promoter, neighboring protein-coding genes, or more distal genes (Wei et al. 2011). According to this model and based on the fact that APOLLO gene is heterozygote (for both Apo- and Sex-allele), there is a possibility that transcription starts from a point in the Apo-allele promoter and continues toward the Sex-allele or vice versa and consequently gives rise to variable transcriptions for apomictic *Boechera.*

The difference in GUS activities observed between the 2 kb Apo vs. Sex native promoter constructs may also be influenced by structural variations that selectively bind particular regulatory elements, leading to varying levels of expression. Furthermore, protein diversity between APOLLO alleles could arise through alternative splicing, in which a variety of exon combinations are used to generate multiple mRNAs from a single gene (Pajares et al., 2007; Davuluri et al., 2008). An important example is the hemoglobin γ A gene (*HBG1*) with TATA-box-lacking and TATA-box-containing alternative promoters that are used at different developmental stages (Duan et al. 2002). In this way, variation between APOLLO Apo- and Sex-alleles could have accumulated after a loss in recombination capacity as explained by the Meselson effect (Hojsgaard and Hörandl 2015). This diversity has been fixed in some motifs such as the 20 bp insertion of Apo-allele, and could produce differential gene expression in a mechanism like that of alternative promoters.

Concluding remarks and future perspectives

Synthetic promoters can drive constitutive, spatiotemporal, inducible, and even unique combinations of transgene expression patterns, depending on the included elements. All synthetic promoters used in this experiment generated unique spatiotemporal expression patterns induced by the combination of elements used in their structures. In conclusion, future studies are required to shed light on the mechanism of tissue-specific transcription activation and/or

repression; the yeast one-hybrid assay and EMSA experiment might reveal the transcription factor machinery interacting with the promoter of APOLLO gene. In addition, it is helpful to know if there is any interaction with tissue-specific transcriptional cofactors that either activate or repress expression or may function in a combinatorial manner with other TFs. Finally, the complex regulatory architecture of apomixis could also include non-coding RNAs (ncRNA) and bidirectional promoters, and thus understanding whether the APOLLO has bidirectional promoter activities in sexual and apomictic *Boechera* would provide further insights into its regulation.

References

Alexander, P. J., Windham, M. D., Beck, J. B., Al-Shehbaz, I. A., Allphin, L, Bailey, C. D (2013). Molecular phylogenetics and taxonomy of the genus *Boechera* and related genera (Brassicaceae: Boechereae). *Systematic Botany* 38(1), 192–209. doi.org/10.1600/036364413X661917.

Aliyu, O. M., Schranz, M. E. and Sharbel, T. F. (2010). Quantitative variation for apomictic reproduction in the genus Boechera (Brassicaceae). *American journal of botany* 97(10), 1719–31. https://doi.org/10.3732/ajb.1000188

Babu, D., Vannelli, S., Antonella, F., Simona, M., Baffico, A. M., Lucia, C., et al. (2021). "Variants in the 5′UTR Reduce SHOX Expression and Contribute to SHOX Haploinsufficiency. *European Journal of Human Genetics* 29(1),110–21. https://doi.org/10.1038/s41431-020-0676-y.

Beck, J. B., Alexander, P. J., Allphin, L., Al-Shehbaz, I. A., Rushworth, C., Bailey, C. D., et al., (2012). Does hybridization drive the transition to asexuality in diploid Boechera? *Evolution* 66(4), 985–995. https://doi.org/10.1111/j.1558-5646.2011.01507.x

Béziat, Ch, Jürgen K-V, and Feraru, E. (2017). Histochemical Staining of β-Glucuronidase and Its Spatial Quantification BT - Plant Hormones: Methods and Protocols. In eds. Jürgen Kleine-Vehn and Michael Sauer. New York, NY: Springer New York, 73–80. https://doi.org/10.1007/978-1-4939-6469-7_8.

Bicknell, R. A., Lambie, S. C., & Butler, R. C. (2003). Quantification of progeny classes in two facultatively apomictic accessions of Hieracium. *Hereditas* 138(1), 11–20. https://doi.org/10.1034/j.1601-5223.2003.01624.x

Bicknell, R., Catanach, A., Hand, M., & Koltunow, A. (2016). Seeds of doubt: Mendel's choice of Hieracium to study inheritance, a case of right plant, wrong trait. *Theoretical and Applied Genetics*. *Springer Verlag* 129(12), 2253–2266. https://doi.org/10.1007/s00122-016-2788-x

Brukhin, V., Osadtchiy, J. V., Florez-Rueda, A. M., Smetanin, D., Bakin, E., Nobre, M. S., et al., (2019). The Boechera genus as a resource for apomixis research. *Frontiers in Plant Science* 10, 392. https://doi.org/10.3389/fpls.2019.00392

Bulankova, P., Akimcheva, S., Fellner, N., & Riha, K. (2013). Identification of Arabidopsis Meiotic Cyclins Reveals Functional Diversification among Plant Cyclin Genes. *PLoS Genetics* 9(5), e1003508. https://doi.org/10.1371/journal.pgen.1003508

Carman, J. G. (2001). The gene effect: genome collisions and apomixis. The Flowering of Apomixis: from Mechanisms to Genetic Engineering. 95-110.

Carman, J. G. (1997). Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. *Biological Journal of the Linnean Society* 61(1), 51–94. https://doi.org/10.1111/j.1095-8312.1997.tb01778.x

Cory A, C. H., King, E, J., Jordan, J. R., and Drews. G. N. (1997). Megagametogenesis in Arabidopsis Wild Type and the Gf Mutant. *Sexual Plant Reproduction* 10(1): 49–64. https://doi.org/10.1007/s004970050067.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium -mediated transformation of Arabidopsis thaliana. *The Plant Journal* 16(6), 735–743. https://doi.org/10.1046/j.1365-313x.1998.00343.x

Corral, J. M., Vogel, H., Aliyu, O. M., Hensel, G., Thiel, T., Kumlehn, J., & Sharbel, T. F. (2013). A conserved apomixis-specific polymorphism is correlated with exclusive exonuclease expression in premeiotic ovules of apomictic Boechera species. *Plant Physiology* 163(4), 1660–1672. https://doi.org/10.1104/pp.113.222430

D., Gregory Brian. (2018). Shedding some blue light on alternative promoter usage in plants. *PNAS* 115(30), 7654–56. https://doi.org/10.1073/pnas.1809312115.

D'Erfurth, I., Jolivet, S., Froger, N., Catrice, O., Novatchkova, M., & Mercier, R. (2009). Turning meiosis into mitosis. *PLoS Biology* 7(6), e1000124. https://doi.org/10.1371/journal.pbio.1000124

Darlington, C. D. (1931). Meiosis in diploid and tetraploid Primula sinensis. *Journal of Genetics* 24(1), 65–96. https://doi.org/10.1007/BF03020823

Davuluri, R. V., Suzuki, Y., Sugano, S., Plass, C., & Huang, T. H. M. (2008). The functional consequences of alternative promoter use in mammalian genomes. *Trends in Genetics* 24(4), 167-177. https://doi.org/10.1016/j.tig.2008.01.008

Deng, W., & Blobel, G. A. (2017). Detecting Long-Range Enhancer-Promoter Interactions by Quantitative Chromosome Conformation Capture. *Methods in molecular biology (Clifton, N.J.)* 1468, 51–62. https://doi.org/10.1007/978-1-4939-4035-6_6

Dobeš, Ch., Marcus A. K., Sharbel, T. F. (2006). Embryology, karyology, and modes of reproduction in the North American genus Boechera (Brassicaceae): A compilation of seven decades of research. *Annals of the Missouri Botanical Garden.* 93(3): 517–34. doi:10.3417/0026-6493(2007)93[517:EKAMOR]2.0.CO;2

Fehrer, J., Gemeinholzer, B., Chrtek, J., Bräutigam, S. (2007). Incongruent plastid and nuclear DNA phylogenies reveal ancient intergeneric hybridization in Pilosella hawkweeds (Hieracium, Cichorieae, Asteraceae). *Molecular Phylogenetics and Evolution* 42(2), 347–361. https://doi.org/10.1016/j.ympev.2006.07.004

Galderisi, U., Jori, F. P. Giordano, A. (2003). Cell cycle regulation and neural differentiation. *Oncogene* 22(33), 5208–5219. https://doi.org/10.1038/sj.onc.1206558

Harkess, A., Huang, K., van der Hulst, R., Tissen, B., Caplan, J. L., Koppula, A., et al. (2020). Sex determination by two Y-Linked genes in garden Asparagus. *The Plant cell* 32(6), 1790–1796. https://doi.org/10.1105/tpc.19.00859

Hojsgaard, Diego. (2018). Transient Activation of Apomixis in Sexual Neotriploids May Retain Genomically Altered States and Enhance Polyploid Establishment. *Frontiers in Plant Science* 9. https://www.frontiersin.org/article/10.3389/fpls.2018.00230.

Hojsgaard, D., Hörandl, E. (2015). A little bit of sex matters for genome evolution in asexual plants. *Frontiers in Plant Science* 6, 82. https://doi.org/10.3389/fpls.2015.00082

Hojsgaard, D., Hörandl, E. (2019). The Rise of Apomixis in natural plant populations. *Frontiers in Plant Science* 10. https://www.frontiersin.org/article/10.3389/fpls.2019.00358.

James, A. B., Sullivan, S., Nimmo, H. G. (2018). Global spatial analysis of Arabidopsis natural variants implicates 5′UTR splicing of LATE ELONGATED HYPOCOTYL in responses to temperature. *Plant, Cell & Environment* 41(7), 1524–38. https://doi.org/10.1111/pce.13188.

Kantama, L., Sharbel, T. F., Schranz, M. E., Mitchell-Olds, T., de Vries, S., de Jong, H. (2007). Diploid apomicts of the Boechera holboellii complex display large-scale chromosome substitutions and aberrant chromosomes. *PNAS* 104(35), 14026–14031. https://doi.org/10.1073/pnas.0706647104.

Kiefer, Ch., Dobeš, ch., Sharbel, T.F., Marcus A. Koch. (2009). Phylogeographic structure of the chloroplast DNA gene pool in North American Boechera – a genus and continental-wide perspective. *Molecular Phylogenetics and Evolution* 52(2), 303–11. https://www.sciencedirect.com/science/article/pii/S1055790309000694.

Koltunow, A. M. G., Johnson, S. D., Rodrigues, J. C. M., Okada, T., Hu, Y., Tsuchiya, T., Wilson, S., et al. (2011). Sexual reproduction is the default mode in apomictic Hieracium subgenus Pilosella, in which two dominant loci function to enable apomixis. *Plant Journal* 66(5), 890–902. https://doi.org/10.1111/j.1365-313X.2011.04556.x

Lieberman-Aiden, E., Van Berkum, N. L., Williams, L., Imakaev, M., Ragoczy, T., Telling, A., et al. (2009). Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* 326(5950), 289–293. https://doi.org/10.1126/science.1181369

Lovell, J. T., Aliyu, O. M., Mau, M., Schranz, M. E., Koch, M., Kiefer, C., et al. (2013). On the origin and evolution of apomixis in Boechera. *Plant reproduction* 26(4), 309–315. https://doi.org/10.1007/s00497-013-0218-7

Mau, M., Liiving, T., Fomenko, L., Goertzen, R., Paczesniak, D., Böttner, L., et al. (2021). The spread of infectious asexuality through haploid pollen. *New Phytologist* 230(2), 804–820. https://doi.org/10.1111/nph.17174

Mittelsten Scheid, O. (2022). Mendelian and Non-Mendelian Genetics in Model Plants. *The Plant Cell* koac070. https://doi.org/10.1093/plcell/koac070.

Van Mourik, H,. Van Dijk, A. D. J., Stortenbeker, N., Angenen, G. C., Bemer, M. (2017). Divergent Regulation of Arabidopsis SAUR Genes: A Focus on the SAUR10-Clade. *BMC Plant Biology* 17(1), 245. https://doi.org/10.1186/s12870-017-1210-4.

Okada, T. et al. (2013). Enlarging Cells Initiating Apomixis in Hieracium Praealtum transition to an embryo sac program prior to entering mitosis. *Plant Physiology* 163(1), 216- 231. http://www.plantphysiol.org/content/163/1/216.abstract.

Renner, S. S., Niels A. M. (2021). Plant Sex Chromosomes defy evolutionary models of expanding recombination suppression and genetic degeneration. *Nature Plants* 7(4), 392–402. https://doi.org/10.1038/s41477-021-00884-3.

Rojek, J., Kapusta, M., Kozieradzka-Kiszkurno, M., Majcher, D., Górniak, M., Sliwinska, E., Sharbel, T. F., & Bohdanowicz, J. (2018). Establishing the cell biology of apomictic reproduction in diploid Boechera stricta (Brassicaceae). Annals of botany, 122(4), 513–539. https://doi.org/10.1093/aob/mcy114

Rushworth, C. A., Song, B. H., Lee, C. R., & Mitchell-Olds, T. (2011). Boechera, a model system for ecological genomics. Molecular Ecology. 20(23), 4843-4857. https://doi.org/10.1111/j.1365-294X.2011.05340.x

Rushworth, C. A., Windham, M. D., Keith, R. A., Mitchell-Olds. T. (2018). Ecological differentiation facilitates fine-scale coexistence of sexual and asexual Boechera. *American Journal of Botany* 105(12), 2051–64. https://doi.org/10.1002/ajb2.1201.

Schmidt, A. (2020). Controlling Apomixis: shared features and distinct characteristics of gene regulation. *Genes* 11(3), 329. https://pubmed.ncbi.nlm.nih.gov/32245021.

Smyth, D. R., Bowman, J. L., Meyerowitz. E. M. (1990). Early flower development in Arabidopsis. *The Plant cell* 2(8), 755–67. https://pubmed.ncbi.nlm.nih.gov/2152125.

Spillane, Ch., Curtis, M. D., Grossniklaus, U. (2004). Apomixis technology development—Virgin Births in Farmers' Fields? *Nature Biotechnology* 22(6), 687–91. https://doi.org/10.1038/nbt976.

Srivastava, A. K., Lu, Y., Zinta, G., Lang, Zh., Zhu, J-K. (2018). UTR-Dependent control of gene expression in pPlants. *Trends in plant science* 23(3), 248–59. https://pubmed.ncbi.nlm.nih.gov/29223924.

Trinklein, N D et al. (2004). An Abundance of Bidirectional Promoters in the Human Genome. *Genome research* 14(1), 62–66. https://pubmed.ncbi.nlm.nih.gov/14707170.

Tucker, M. R., Koltunow, A. M. G. (2009). Sexual and Asexual (Apomictic) Seed Development in Flowering Plants: Molecular, Morphological and Evolutionary Relationships. *Functional Plant Biology* 36(6), 490–504.

Underwood, Ch. J. et al. (2022). A PARTHENOGENESIS Allele from Apomictic Dandelion Can Induce Egg Cell Division without Fertilization in Lettuce." *Nature Genetics* 54(1), 84–93. https://doi.org/10.1038/s41588-021-00984-y.

Underwood, Ch. J., Vijverberg, K., Rigola, D., Okamoto, Sh., Oplaat, C., Op den Camp, R. H. M., et al. (2022). Engineering Apomixis: Clonal Seeds Approaching the Fields. *Annual Review of Plant Biology*. https://doi.org/10.1146/annurev-arplant-102720-013958.

Vernet, A., Meynard, D., Lian, Q., Mieulet, D., Gibert, O., Bissah, M., et al. (2022). High-Frequency Synthetic Apomixis in Hybrid Rice. *Nature Communications* 13(1), 7963. https://doi.org/10.1038/s41467-022-35679-3.

Wu, W., Pelechano, V., Järvelin, A. I., Steinmetz, L. M. (2011). Functional Consequences of Bidirectional Promoters. *Trends in Genetics* 27(7), 267–76. http://www.sciencedirect.com/science/article/pii/S0168952511000588.

Westergaard, M. (1958). The Mechanism of Sex Determination in Dioecious Flowering Plants. In ed. M B T - Advances in Genetics *Demerec. Academic Press* 217–81. https://www.sciencedirect.com/science/article/pii/S0065266008601637.

Dossa, K., Zhou, R., Li , D., Liu, A., Quin, L., A Mmadi, M., et al. (2021). A Novel Motif in the 5'-UTR of an Orphan Gene 'Big Root Biomass' Modulates Root Biomass in Sesame. *Plant Biotechnology Journal* 19(5), 1065–79. https://doi.org/10.1111/pbi.13531.

Duan, Z. J., Fang, X., Rohde, A., Han, H., Stamatoyannopoulos, G., Li, Q. (2002). Developmental specificity of recruitment of TBP to the TATA box of the human gamma-globin gene. *Proceedings of the National Academy of Sciences of the United States of America.* 99(8), 5509–5514. https://doi.org/10.1073/pnas.072084499

Erdmann, R. M., Hoffmann, A., Walter, H. K., Wagenknecht, H. A., Groß-Hardt, R., & Gehring, M. (2017). Molecular movement in the Arabidopsis thaliana female gametophyte. *Plant reproduction* 30(3), 141–146. https://doi.org/10.1007/s00497-017-0304-3

Fehrer, J., Gemeinholzer, B., Chrtek, J., Bräutigam, S. (2007). Incongruent plastid and nuclear DNA phylogenies reveal ancient intergeneric hybridization in Pilosella hawkweeds (Hieracium, Cichorieae, Asteraceae). *Molecular Phylogenetics and Evolution* 42(2), 347–361. https://doi.org/10.1016/j.ympev.2006.07.004

Galderisi, U., Jori, F. P. Giordano, A. (2003). Cell cycle regulation and neural differentiation. *Oncogene* 22(33), 5208–5219. https://doi.org/10.1038/sj.onc.1206558

Harkess, A., Huang, K., van der Hulst, R., Tissen, B., Caplan, J. L., Koppula, A., et al. (2020). Sex determination by two Y-Linked genes in garden Asparagus. *The Plant cell* 32(6), 1790–1796. https://doi.org/10.1105/tpc.19.00859

Harrison S. J., Mott E.K., Parsley, K., Aspinall, S., Gray, J.C., Cottage A., (2006). A rapid and robust method of identifying transformed Arabidopsis Thaliana seedlings following floral dip transformation. *Plant methods* 2: 19. https://pubmed.ncbi.nlm.nih.gov/17087829.

Hojsgaard, Diego. (2018). Transient Activation of Apomixis in Sexual Neotriploids May Retain Genomically Altered States and Enhance Polyploid Establishment. *Frontiers in Plant Science* 9. https://www.frontiersin.org/article/10.3389/fpls.2018.00230.

Hojsgaard, D., Hörandl, E. (2015). A little bit of sex matters for genome evolution in asexual plants. *Frontiers in Plant Science* 6, 82. https://doi.org/10.3389/fpls.2015.00082

Hojsgaard, D., Hörandl, E. (2019). The Rise of Apomixis in natural plant populations. *Frontiers in Plant Science* 10. https://www.frontiersin.org/article/10.3389/fpls.2019.00358.

James, A. B., Sullivan, S., Nimmo, H. G. (2018). Global spatial analysis of Arabidopsis natural variants implicates 5′UTR splicing of LATE ELONGATED HYPOCOTYL in responses to temperature. *Plant, Cell & Environment* 41(7), 1524–38. https://doi.org/10.1111/pce.13188.

Kantama, L., Sharbel, T. F., Schranz, M. E., Mitchell-Olds, T., de Vries, S., de Jong, H. (2007). Diploid apomicts of the Boechera holboellii complex display large-scale chromosome substitutions and aberrant chromosomes. *PNAS* 104(35), 14026–14031. https://doi.org/10.1073/pnas.0706647104.

Kiefer, Ch., Dobeš, ch., Sharbel, T.F., Marcus A. Koch. (2009). Phylogeographic structure of the chloroplast DNA gene pool in North American Boechera – a genus and continental-wide perspective. *Molecular Phylogenetics and Evolution* 52(2), 303–11. https://www.sciencedirect.com/science/article/pii/S1055790309000694.

Koltunow, A. M. G., Johnson, S. D., Rodrigues, J. C. M., Okada, T., Hu, Y., Tsuchiya, T., Wilson, S., et al. (2011). Sexual reproduction is the default mode in apomictic Hieracium subgenus Pilosella, in which two dominant loci function to enable apomixis. *Plant Journal* 66(5), 890–902. https://doi.org/10.1111/j.1365-313X.2011.04556.x

Li, Ch., Xu, P., Zhou, A., Song, J., Wu., Y. He, T. (2022). A preliminary study of Apomictic characteristics of 'Mianli' (Pyrus Sinkiangensis Yü). *Hort Science* 57(7), 818–26. https://journals.ashs.org/hortsci/view/journals/hortsci/57/7/article-p818.xml.

Lieberman-Aiden, E., Van Berkum, N. L., Williams, L., Imakaev, M., Ragoczy, T., Telling, A., et al. (2009). Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* 326(5950), 289–293. https://doi.org/10.1126/science.1181369

Lovell, J. T., Aliyu, O. M., Mau, M., Schranz, M. E., Koch, M., Kiefer, C., et al. (2013). On the origin and evolution of apomixis in Boechera. *Plant reproduction* 26(4), 309–315. https://doi.org/10.1007/s00497-013-0218-7

Luo, K. R., Huang, N. C., Yu, T. S. (2018). Selective targeting of mobile mRNAs to plasmodesmata for Cell-to-Cell movement. *Plant Physiology* 177(2), 604 – 614. https://doi.org/10.1104/pp.18.00107

Mau, M., Lovell, J. T., Corral, J. M., Kiefer, C., Koch, M. A., Aliyu, O. M., et al. (2015). Hybrid apomicts trapped in the ecological niches of their sexual ancestors. *PNAS* 112(18), 2357–2365. https://doi.org/10.1073/pnas.1423447112

Mau, M., Liiving, T., Fomenko, L., Goertzen, R., Paczesniak, D., Böttner, L., et al. (2021). The spread of infectious asexuality through haploid pollen. *New Phytologist* 230(2), 804–820. https://doi.org/10.1111/nph.17174

Mittelsten Scheid, O. (2022). Mendelian and Non-Mendelian Genetics in Model Plants. *The Plant Cell* koac070. https://doi.org/10.1093/plcell/koac070.

van Mourik, H. et al. (2017). Divergent Regulation of Arabidopsis SAUR Genes: A Focus on the SAUR10-Clade. *BMC Plant Biology* 17(1), 245. https://doi.org/10.1186/s12870-017-1210-4.

Okada, T. et al. (2013). Enlarging Cells Initiating Apomixis in Hieracium Praealtum transition to an embryo sac program prior to entering mitosis. *Plant Physiology* 163(1), 216- 231. http://www.plantphysiol.org/content/163/1/216.abstract.

Renner, S. S., Niels A. M. (2021). Plant Sex Chromosomes defy evolutionary models of expanding recombination suppression and genetic degeneration. *Nature Plants* 7(4), 392–402. https://doi.org/10.1038/s41477-021-00884-3.

Lee, Ch., MITCHELL-OLDS, Th. (2011). Boechera, a Model System for Ecological Genomics. *Molecular Ecology* 20(23),4843–57. https://doi.org/10.1111/j.1365-294X.2011.05340.x.

Rushworth, C. A., Windham, M. D., Keith, R. A., Mitchell-Olds. T. (2018). Ecological differentiation facilitates fine-scale coexistence of sexual and asexual Boechera. *American Journal of Botany* 105(12), 2051–64. https://doi.org/10.1002/ajb2.1201.

Schmidt, A. (2020). Controlling Apomixis: shared features and distinct characteristics of gene regulation. *Genes* 11(3), 329. https://pubmed.ncbi.nlm.nih.gov/32245021.

Smyth, D. R., Bowman, J. L., Meyerowitz. E. M. (1990). Early flower development in Arabidopsis. *The Plant cell* 2(8), 755–67. https://pubmed.ncbi.nlm.nih.gov/2152125.

Spillane, Ch., Curtis, M. D., Grossniklaus, U. (2004). Apomixis technology development—Virgin Births in Farmers' Fields? *Nature Biotechnology* 22(6), 687–91. https://doi.org/10.1038/nbt976.

Srivastava, A. K., Lu, Y., Zinta, G., Lang, Zh., Zhu, J-K. (2018). UTR-Dependent control of gene expression in pPlants. *Trends in plant science* 23(3), 248–59. https://pubmed.ncbi.nlm.nih.gov/29223924.

Tilsner, J., William, N., Abel, R., Emmanuelle, M. B. (2016). Staying Tight: Plasmodesmal Membrane Contact Sites and the Control of Cell-to-Cell Connectivity in Plants. *Annual Review of Plant Biology*

67(1), 337–64. https://doi.org/10.1146/annurev-arplant-043015-111840.

Trinklein, N D et al. (2004). An Abundance of Bidirectional Promoters in the Human Genome. *Genome research* 14(1), 62–66. https://pubmed.ncbi.nlm.nih.gov/14707170.

Tucker, M. R., Koltunow, A. M. G. (2009). Sexual and Asexual (Apomictic) Seed Development in Flowering Plants: Molecular, Morphological and Evolutionary Relationships. *Functional Plant Biology* 36(6), 490–504.

Underwood, Ch. J. et al. (2022). A PARTHENOGENESIS Allele from Apomictic Dandelion Can Induce Egg Cell Division without Fertilization in Lettuce." *Nature Genetics* 54(1), 84–93. https://doi.org/10.1038/s41588-021-00984-y.

Underwood, Ch. J., Vijverberg, K., Rigola, D., Okamoto, Sh., Oplaat, C., Op den Camp, R. H. M., et al. (2022). Engineering Apomixis: Clonal Seeds Approaching the Fields. *Annual Review of Plant Biology*. https://doi.org/10.1146/annurev-arplant-102720-013958.

Vernet, A., Meynard, D., Lian, Q., Mieulet, D., Gibert, O., Bissah, M., et al. (2022). High-Frequency Synthetic Apomixis in Hybrid Rice. *Nature Communications* 13(1), 7963. https://doi.org/10.1038/s41467-022-35679-3.

Wu, W., Pelechano, V., Järvelin, A. I., Steinmetz, L. M. (2011). Functional Consequences of Bidirectional Promoters. *Trends in Genetics* 27(7), 267–76. http://www.sciencedirect.com/science/article/pii/S0168952511000588.

Westergaard, M. (1958). The Mechanism of Sex Determination in Dioecious Flowering Plants. In ed. M B T - Advances in Genetics *Demerec. Academic Press* 217–81. https://www.sciencedirect.com/science/article/pii/S0065266008601637.

Kurihara, Y., Makita, Y., Kawashima, M., Matsui, M. (2018). Transcripts from Downstream Alternative Transcription Start Sites Evade UORF-Mediated Inhibition of Gene Expression in Arabidopsis. *Proceedings of the National Academy of Sciences* 115(30), 7831–36. https://doi.org/10.1073/pnas.1804971115.