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## Introduction

Alternate bearing (AB), a biennial fluctuation of crop yield, is a major hindrance for the pecan industry. Fluctuation in yields is often associated with variation in nut quality, in which higher croploads are likely to result in smaller, lower-valued nuts and vice versa. Inconsistencies in yields from year to year tend to be synchronized not only from tree to tree within an orchard, but between orchards, and even across the entire US pecan growing belt. Synchronized AB impedes the pecan industry by disrupting growers' budgets, creating unbalanced supply and demand levels for labor and equipment, and influencing product pricing and availability.

Pecan flowers are imperfect with staminate (male) "catkins" consisting of long inflorescences forming in clusters of three on previous season lateral buds (Fig. 1). The pistillate (female) flowers, lacking petals and sepals, are borne on spikes at the terminal end of current season shoot growth (Fig. 1).

Identification of the location and timing of molecular floral initiation signals within pecan tissues is key to the understanding of AB and eventual mitigation of AB in pecan. In research on the model plant *Arabidopsis thaliana*, major advancements have led to isolation and characterization of over 180 genes involved in flowering.

Exogenous applications of plant growth regulators (PGRs) can potentially be used as tools for modification of flowering behavior and mitigation of alternate bearing in pecan, as well as in other species such as apple and citrus. However, connections between effects of PGR applications and expression levels of genes likely to be involved with flowering have not been previously examined in pecan. The objective of this study was to identify differences in expression of pecan homologs of the flowering genes *LFY* (*CpLFY*), *AP1* (*CpAP1*), and *FT* (*CpFT*) in leaf and bud tissues selected from fruiting and non-fruiting shoots on 'Western' pecan trees over time and after exogenous applications of various PGRs.

**Figure 1.** Pecan floral structures. Staminate inflorescences (catkins) hanging in long spikes clustered in three and borne from lateral buds on previous season shoot (left). Cluster of four pistillate flowers borne on terminal end of a current season (new) shoot (right).

## Materials and Methods

***Experimental Location and Design***

- 45-year-old commercially managed, flood-irrigated pecan orchard in Mesilla Valley, New Mexico, United States (Fig. 2)
- Six Western cultivar pecan trees were selected for this study from a single irrigation plot.
- Randomized complete block design

***Exogenous Application of Plant Growth Regulator Treatments***

- Plant growth regulators (PGRs) were applied 3 times per season, at approximately 3-, 6-, and 9- weeks after full bloom, to current season individual shoots in two growing seasons: 2014 and 2015 (Fig. 3)
- Return bloom data was collected from the treated shoots in springs of 2015 and 2016.
- Treatments (each with an added surfactant)

  In 2014, 3 treatments were applied to both fruiting and non-fruiting shoot populations:

  1) Control
  2) Ethephon (as Ethepon2® [100 mg·L$^{-1}$ a.i.])
  3) Gibberellic acid ($GA_3$; as ProGibb® [50 mg·L$^{-1}$ a.i.])

  In 2015 two additional treatments were:

  4) 2X gibberellic acid (as ProGibb® [100 mg·L$^{-1}$ a.i.])
  5) Aminoethoxyvinylglycine (AVG; as ReTain® [88 mg·L$^{-1}$ a.i.])

***Tissue Sampling***

- Approximately 1 week post application of PGRs a whole leaf and the associated bud were sampled from 1 non-fruiting shoot and 1 fruiting shoot per treatment group in each tree
- Total of 3 sample dates and 108 total shoots sampled from this site in 2014 and 180 shoots sampled in 2015. The samples were maintained at -80°C until further processing

***Gene Expression Analyses of Tissues***

- Leaf and bud tissues were homogenized separately and approximately 100 mg of ground tissue was utilized for RNA isolation with Purelink® Plant RNA Reagent (Invitrogen, Carlsbad, CA)
- RNA from each sample was treated with DNase to remove any contaminant DNA (Fig. 4)
- DNase-treated RNA was quantified using a NanoDrop 1000 spectrophotometer
- Complementary DNA (cDNA) synthesized using a SuperScript® IV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA)
- Gene specific primers were designed for LFY and actin using nucleotide alignments of hickory, walnut, apple, and poplar with Geneious v 5.5.7
- The pecan homologs *CpAP1* and *CpFT* were identified using the pecan draft genome 87 MX 3.11 (Jenkins, 2013) using Geneious 9.1 software (Kearse et al., 2012) (Table 1)
- Primer probes assays were synthesized by Integrated DNA Technologies (Coralville, IA) each with a unique fluorescent reporter dye and a quencher (Table 2)
- Assays for each sample were performed in triplicate using iQ™ Multiplex Powermix (Bio-Rad)
- The CFX96 touch real-time detection system (BioRad, Hercules, CA) was utilized and the qRT-PCR program was 95°C for 2.5 min, followed by 39 cycles of 95°C for 15 s, and 60°C for 60 s
- Each qRT-PCR result was regarded as positive for any cycle threshold value less than 35.0
- Starting quantities (SQ) were calculated by BioRad software using the CFX Touch Real-Time PCR Detection System
- For each sample, the average target gene SQ from three technical replicates performed within each plate were normalized to the reference gene SQ (*CpACTIN*). Therefore, normalized gene expression was calculated as the ratio of average target gene SQ to average reference gene SQ

***Data Analysis***

- Gene expression variables were analyzed using a mixed model with fixed effects for treatment, fruiting status, date and all interactions among these three factors
- The model incorporated random effects corresponding to the blocking factor, tree, and tree x date
- Fisher's least significant difference (LSD) was used for *post hoc* pair-wise comparisons applied to assess simple effects corresponding to the highest order significant effect
- Analyses were executed using SAS version 9.4 software (copyright © 2012 SAS Institute Inc., Cary, NC, USA)
- Significance, $\alpha = 0.05$ for all analyses. Differences were acknowledged as "marginally significant" when $\alpha = 0.10$
- For all analyses the outlier strategy was employed with outliers defined as data points producing studentized residuals with magnitudes greater than three

**Figure 2.** Selecting shoots in mature pecan tree canopy.

**Figure 3.** Spray application of plant growth regulator on non-fruiting pecan shoot.

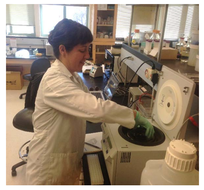

**Figure 4.** Centrifugation step during isolation of RNA.

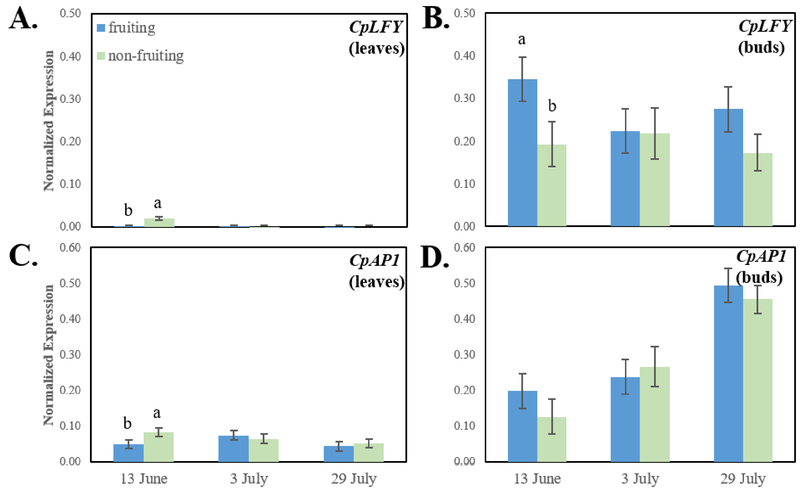


**Figure 5.** Seasonal expression profiles of 2014 *CpLFY* and *CpAP1* in control group only. Seasonal expression profiles of *CpLFY* (A, B) and *CpAP1* (C, D) in leaves (A, C) and buds (B, D) from control group fruiting (on) and non-fruiting (off) pecan shoots in 2014 are shown. Data are lsmeans ± $_{SE}$ of six independent replicates (n = 6 trees) and three technical replicates. A significant difference between the normalized expression in tissues from fruiting and non-fruiting shoots at the same time point is denoted with lowercase letters when $P \leq 0.05$.

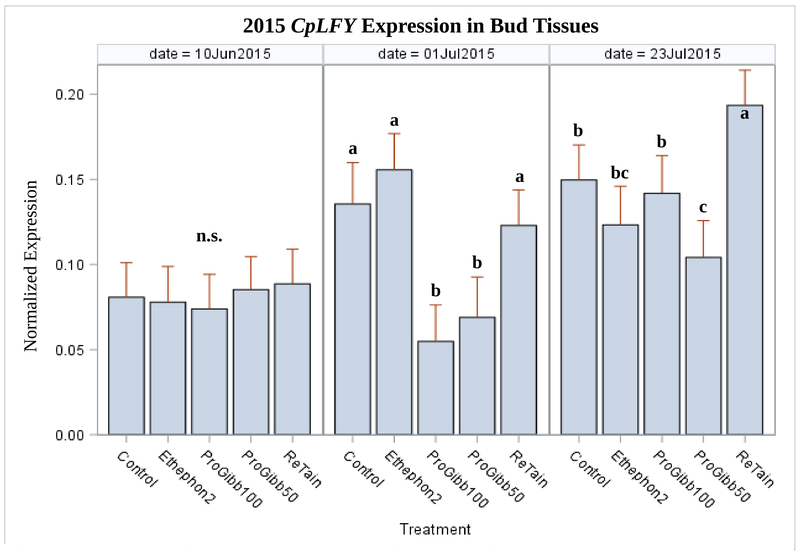


**Figure 6.** Pecan bud tissue *CpLFY* normalized expression in 2015. LSMEANS, organized by date and treatment group, are shown with error bars depicting standard errors. Different lowercase letters represent statistically different lsmeans within that date ($P \leq 0.05$).

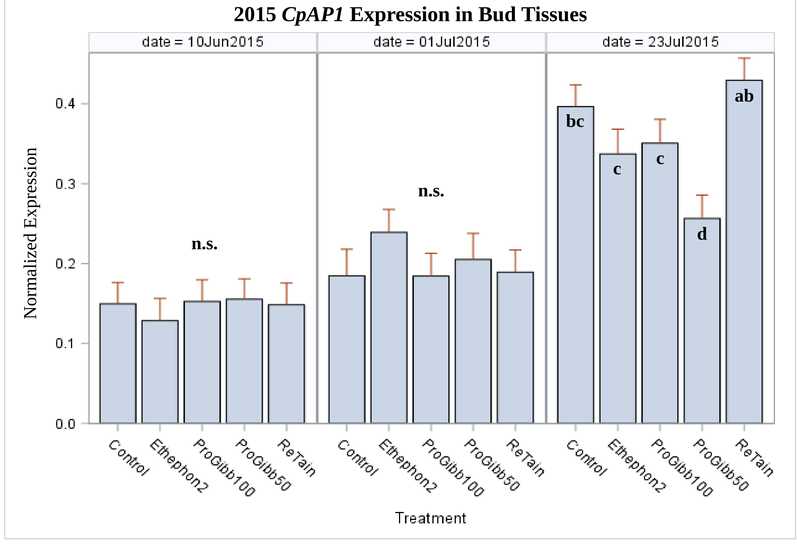


**Figure 7.** *CpAP1* gene expression in 2015 bud tissues by treatment and date.

**Table 1.** Primers and probes used for q-PCR analysis. Gene targets, identifiers, and sequences shown.

| Target Gene | Assay Form | Sequence |
|---|---|---|
| *CpACTIN* | Probe | 5'-/5HEX/TGGAAGAGA/ZEN/ACTTCTGGGCAACGG/3IABkFQ/-3' |
| | Primer 1 | 5'-TTGTATGTGGTCTCGTGGATTC-3' |
| | Primer 2 | 5'-ATCACAATTGGAGCTGAGAGG-3' |
| *CpLFY* | Probe | 5'-/56-FAM/AGCAGTGCC/ZEN/GTGATTTCTTGATCCA/3IABkFQ/-3' |
| | Primer 1 | 5'-CGCTCCTTCGCTATGTTCTG-3' |
| | Primer 2 | 5'-CGATTACCTCTTCCATCTCTACG-3' |
| *CpAP1* | Probe | 5'-/5TEX615/TGCGCTTTCTTCAGCAAACCAGAC/3IAbRQSp/-3' |
| | Primer 1 | 5'-AGAGGATCGAGAACAAGATCAA-3' |
| | Primer 2 | 5'-GCATCACAAAGCACAGAGAT-3' |
| *CpFT* | Probe | 5'-/5Cy5/AGCTCAAGCCGTCTCATGTTGTCA/3IAbRQSp/-3' |
| | Primer 1 | 5'-CTCTGAGGGTCACTTACAACAA-3' |
| | Primer 2 | 5'-GTCATCACCGCCTACATCAA-3' |

**Table 2.** Traits of probe fluorophores.

| Target Gene | Fluorophore (Dye) | Excitation (nm) | Detection (nm) |
|---|---|---|---|
| *CpACTIN* | HEX™ | 515-535 | 560-580 |
| *CpLFY* | 6-FAM™ | 450-490 | 515-530 |
| *CpAP1* | Texas Red™ | 560-590 | 610-650 |
| *CpFT* | Cy®5 | 620-650 | 675-690 |

## Results

***Gene Expression in Untreated Control Samples***

- In the control treatment bud means for normalized gene expression were 0.233 ± 0.0210 and 0.113 ± 0.0132 in 2014 and 2015, respectively.
- In the control samples, significant differences in *CpLFY* expression between fruiting and non-fruiting shoots were only detected on 13 June 2014 in leaf tissues (Fig 5A) and bud tissues (Fig. 5B).
- *CpAP1* expression in non-fruiting shoots was significantly higher than in fruiting shoots in the leaf tissues from untreated shoots on 13 June 2014, (Fig. 5C).
- Fruiting status effect on *CpAP1* was not detected in bud tissues, however a date effect is apparent in which *CpAP1* levels appear to be increasing into late July (Fig. 5D).

***Gene Expression Response to Tissue Type, Fruiting Status, Sampling Date, and Plant Growth Regulator Treatments***

- *CpLFY* was more highly expressed in bud tissue than in leaves with 97.63% of leaf samples in 2015 showing no expression of *CpLFY* at all. *CpLFY* expression in the leaf tissues displayed no significant differences based on shoot fruiting status, sampling date, or PGR treatment in either year.
- In the bud tissues, *CpLFY* expression differed significantly by sampling date in both years.
- In 2015, a two-way interaction of treatment by date ($P$=0.0008) was found in 2015 in bud tissue *CpLFY* expression levels with no differences due to treatment found on 10 June, a reduction of bud *CpLFY* expression in both of the GA3 treatments on 1 July, and on 23 July an increase in expression in the ReTain treatment as well as a decrease in expression due to the ProGibb100 $GA_3$ treatment (Fig. 6).
- In 2015 date main effect was significant with 10 June *CpAP1* expression values higher than those on both 1 July and 23 July.
- In 2015 a significant fruiting by date interaction was detected with buds from fruiting shoots expressing *CpAP1* at higher rates than in non-fruiting shoots and again with the latest date, 23 July, having higher expression rates than the other two dates (Fig. 7).
- In 2015 bud *CpAP1* interaction of treatment by date interaction was also significant.
- Because *CpFT* was not found to be expressed in 93% and 95% of the bud samples, this variable is not discussed here.

## Conclusions

- With the genes that we evaluated, buds are a far better predictor of return bloom in pecan than leaf tissues.
- We detected significant differences in expression of each of the 3 selected flowering gene homologs (*CpLFY*, *CpAP1*, and *CpFT*) based on shoot fruiting status at specific time points.
- Differences based on PGR treatment in the flowering homologs *CpLFY* and *CpAP1* were detected and we propose that these flowering homologs have a role in pistillate flower initiation.
- Regulatory networks in flowering plants that control floral initiation in response to environmental, endogenous, and autonomous signals are known to be complex, and pecan is no exception.
- PGR treatments affected return bloom, thus, the change in return bloom may be correlated to gene expression.
- We propose a multi-step decision process which dictates pistillate flower initiation in pecan (Fig. 8).

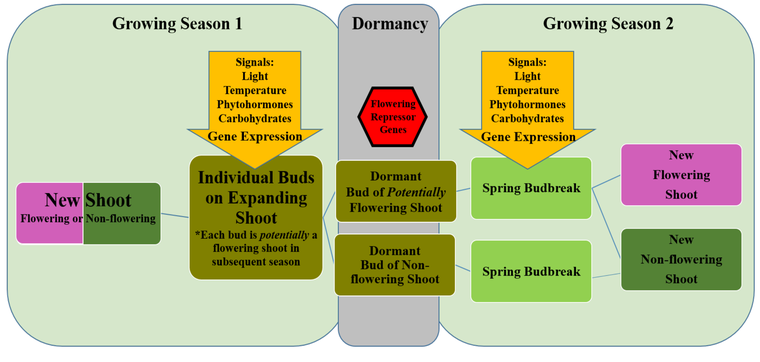


**Figure 8.** Schematic of proposed process for floral initiation in pecan. In this proposed schematic of the process, floral initiation begins early in the growing season of year one with genetic switches that respond to both environmental (light and temperature) and endogenous signals (including phytohormone production and carbohydrate status), which start a cascade of activity and, eventually, up-regulation of flowering gene repressors (likely *FLOWERING LOCUS C*) before and during winter dormancy. Upon spring emergence from dormancy, as budbreak begins, the second phase of the flowering decision process includes those same environmental and endogenous signals which play a role in gene activity (including turning off of flowering gene repressors).

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