Characterization of ethylene-inducible pedicel-fruit abscission zone formation in non-climacteric sweet cherry (*Prunus avium* L.)

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Abstract

Sweet cherry (*Prunus avium* L.) fruit harvest is a labor-intensive process. Mechanical harvesting of sweet cherry fruit is feasible; however, it is dependent on the formation of an abscission zone at the fruit-pedicel junction. The natural propensity for pedicel-fruit abscission zone (PFAZ) formation varies by cultivar, and the general molecular basis for PFAZ formation is not well characterized. In this study, ethylene-inducible change in pedicel fruit retention force (PFRF) was recorded in a developmental time course with a concomitant analysis of the PFAZ transcriptome from three sweet cherry cultivars. In 'Skeena', mean PFRF for both control and treatment fruit dropped below the 0.40kg-force (3.92N) threshold for mechanical harvesting and indicating the formation of a discrete PFAZ. In 'Bing', mean PFRF for both control and treatment groups decreased over time. However, a mean PFRF conducive to mechanical harvesting was achieved only in the ethylene-treated fruit. While in 'Chelan' the mean PFRF of the control and treatment groups did not meet the threshold required for efficient mechanical harvesting. Transcriptome analysis of the PFAZ followed by the functional annotation, differential expression analysis, and gene ontology (GO) enrichment analyses of the data facilitated the identification of phytohormone-responsive and abscission-related transcripts as well as processes that exhibited differential expression and enrichment in a cultivar-dependent manner over the developmental time-course. Additionally, read alignment-based variant calling revealed several short variants within essential ethylene- and auxin-responsive genes, likely explaining differences in phytohormone-responses between the cultivars. These results provide genetic targets for induction or inhibition of PFAZ formation, depending on the desire to harvest the fruit with or without the stem attached. Understanding the genetic mechanisms underlying the development of the PFAZ will inform future cultivar development while laying a foundation for mechanized sweet cherry harvest.

Key Words

Ethephon, ethylene, auxin, RNA-seq, transcriptome, *Prunus avium*, abscission, qRT-PCR, gene expression, Rosaceae

Introduction

Sweet cherry (*Prunus avium* L.), a member of the Rosaceae family, is a commercially important tree fruit crop throughout the world, with approximately 2.3 million tons produced annually (Axe and Bush 2017, Blando and Oomah 2019). In addition to its worldwide economic value, production, and consumption of sweet cherries has increased in recent years as consumers have become aware of their nutritional benefits (Blando and Oomah 2019). The industry is evolving to address the growing market, availability of labor during harvest, and has begun adapting harvesting strategies to meet new consumer demand for stemless cherries (Kappel et al. 2012, Quero-García et al. 2017). Traditional harvesting methods focus on separating the fruit from the tree at the pedicel-peduncle junction, leaving the pedicel (stem) attached to the fruit (Wittenbach and Bukovac 1974b). Mechanical harvesting, on the other hand, is best achieved when the fruit abscises easily at the fruit-pedicel junction (Quero-García, Schuster, López-Ortega and Charlot 2017). Increasing labor costs associated with traditional hand harvesting, in addition to the growing demand for stemless fruit, has made adoption of mechanical harvesting strategies attractive if they can be made uniform across cultivars (Zhou et al., 2016). However, the harvesting of sweet cherries has presented a unique set of challenges. Unlike sour cherries (*Prunus cerasus*), which develop an anatomically and histologically distinct fruit-pedicel abscission zone (PFAZ) and separate with ease, sweet cherry cultivars display phenotypic differences in PFAZ formation and consequent ease of fruit separation—some cultivars require excessive force to separate fruit at the PFAZ, which tends to compromise fruit quality and integrity (Stösser et al. 1969, Zhao et al. 2013).

While sweet cherry and peach belong to the same sub-family, the former bears non-climacteric fruits that do not produce ethylene autocatalytically during ripening and senescence.

This is most likely due to the presence of several stop codon mutations in the ethylene biosynthesis and perception genes in sweet cherry (Koepke et al. 2013). However, sweet cherry fruit from some cultivars displays a novel developmental response to exogenous ethylene application (Hiwasa-Tanase and Ezura 2014). Exogenous ethylene application can induce or enhance PFAZ formation, loosening the fruit, and facilitating efficient mechanical harvesting (Smith and Whiting 2010, Wittenbach and Bukovac 1974a).

Pedicel-fruit retention force (PFRF) is used as a direct measure of PFAZ formation and serves as a metric to determine the mechanical harvestability of the fruit. An average PFRF value of 0.40kg-force (3.92N) is considered the threshold for mechanical harvestability, though this will depend on the actuation method of the harvester. A reduction in PFRF can be induced through the application of ethephon (2-chloroethylephosphonic acid), a commercially available plant growth regulator that is rapidly metabolized to ethylene (Smith and Whiting 2010, Zhao, Athanson, Whiting and Oraguzie 2013). The natural PFRF of sweet cherry varies by cultivar, with some varieties, like ‘Skeena’, exhibiting an auto-abscising phenotype and requiring no exogenous ethylene to induce abscission. Representing an intermediate phenotype, ‘Bing’ can be induced to abscise when ethephon is applied approximately 14 days before harvest (Smith and Whiting 2010, Zhao, Athanson, Whiting and Oraguzie 2013). ‘Chelan’, on the contrary, does not abscise naturally or in the presence of ethephon (Smith and Whiting 2010).

Sweet cherry phenotyping studies have shown that PFRF values remain consistent for these cultivars across multiple years, indicating that the abscission phenotypes are genetically stable and can perhaps be manipulated at the genetic level (Zhao, Athanson, Whiting and Oraguzie 2013). Furthermore, these findings suggest that standardization of PFRF for mechanical harvesting across cultivars is possible if the ideal ethephon or other treatment regimens are determined for

88 individual sweet cherry cultivars. Despite the extensive physiological characterization of PFAZ
89 integrity across sweet cherry varieties, the underlying molecular basis for PFAZ structural
90 differences has not previously been elucidated.

91 As understood from studies in model plant systems such as Arabidopsis and tomato,
92 abscission at the fruit-pedicel junction entails a series of hallmark structural changes: the middle
93 lamella is dissolved by hydrolytic enzymes, such as polygalacturonase and cellulase (Taylor et al.
94 1991); cell walls in the separation layer thicken, and cell wall components become hydrated as a
95 result (Huberman et al. 1983); primary cell walls break down as abscission progresses, resulting
96 in the formation of large intercellular cavities (Tabuchi et al. 2000); and lignin deposits accumulate
97 proximally to the abscission zone, forming part of a peridermal boundary layer that will serve to
98 protect the pedicel scar following fruit separation (Merelo et al. 2017, Tabuchi et al. 1998). This
99 process is thought to operate in a similar manner in fruit crops including apple, peach, and olive
100 although species and cultivar-specific differences, particularly with regards to chemical induction
101 of abscission in these crops, are not yet well understood (Ali et al. 2012, Botton et al. 2011, Gil-
102 Amado and Gomez-Jimenez 2013, Zhu et al. 2011).

103 According to the current model of abscission in plants, the genetic events underlying
104 cellular structure modification at the PFAZ center around an interplay between ethylene and auxin-
105 associated pathways. Binding of ethylene to corresponding receptors (ETRs) in PFAZ cells
106 initiates signal transduction pathways leading to the activation of numerous, ethylene-responsive
107 transcription factors (ERFs), which elicit different modulatory roles. The ethylene response and
108 signaling network ultimately results in the initiation of cell death, reduction in cell wall adhesion,
109 and separation of the fruit from the pedicel (Roberts et al. 2002). Working antagonistically to
110 ethylene is the phytohormone auxin. Auxin's biologically active form, free indole-3-acetic acid

(IAA), decreases the sensitivity of plant organs to ethylene. The genetic and metabolic factors governing auxin homeostasis ensure that an appropriate balance is maintained between free IAA, IAA-conjugates, and auxin degradation during different developmental stages (Meir et al. 2010). A presence of higher levels of free IAA corresponds to inhibited or delayed ethylene-dependent developmental responses like abscission zone formation (Else et al. 2004). Decreasing polar auxin transport across the abscission zone in sweet cherries by girdling methods results in increased fruit abscission (Blanusa et al. 2005). Additionally, in grape, the application of inhibitors of auxin transport has been shown to promote increased abscission (Kühn et al. 2016).

While there is evidence implicating the involvement of numerous ethylene-associated transcription factors and auxin-associated genes in the modulation of PFAZ formation, species-specific modes of action have yet to be resolved (Roberts, Elliott and Gonzalez-Carranza 2002). The induction of PFAZ formation in response to exogenous ethylene application in the non-climacteric fruit sweet cherry represents a unique biological system to elucidate the process of inducible abscission. An improved understanding of the interplay between auxin and ethylene response in sweet cherry will facilitate improved strategies for planned induction or inhibition of PFAZ formation.

To elucidate the molecular bases for differences in abscission phenotypes among sweet cherry cultivars, and to correlate this information with fruit development, time course physiological measurements of the PFRF along with concomitant transcriptome analysis of the PFAZ tissue from ethylene-treated and control ‘Bing’, ‘Skeena’, and ‘Chelan’ was conducted. The hypothesis that cultivar-specific gene expression differences in ethylene- and auxin-responsive pathways are directly correlated to the differences in abscission phenotypes was evaluated. The results of this study reveal potential genetic targets for PFAZ formation in sweet cherry, which are

expected to inform strategies for improving PFAZ phenotypes conducive to different harvesting approaches.

Methods

Plant Material

The sweet cherry trees used in this study are located at Washington State University's Roza Farm, 10 km north of Prosser, Washington, USA (46.2°N, 119.7°). Trees were irrigated weekly from bloom to leaf senescence with low-volume, under-tree, micro-sprinklers, and grown using standard orchard management practices. Trees had an in-row spacing of 2.44 m (8 ft) and between row spacing of 4.27 m (14 ft). Rows were planted in a north-south orientation and trained to a Y-trellis architecture.

Ethephon application

Ethephon (formula 240 g/l [2lbs/gal]) was applied via air-blast sprayer at 3.5 L ha−1 (3 pt A−1) with a 1,871 L ha−1 (200 g A−1) spray volume (Smith and Whiting 2010). Applications and measurements were conducted in three different years (2010, 2013, and 2014). Transcriptome analysis using RNAseq was performed with tissues collected in 2014. Each replication was performed in the same orchard block, using distinct trees within the block. Treatment application was done early in the morning (between 0600 and 0800 hours) to reduce the effects of ethylene evolution from warm temperatures and wind, as previously described (Smith and Whiting 2010).

Optimal ethephon application time for 'Bing' had been established previously as 14 days before harvest (DBH) (Smith and Whiting 2010), or 80% fruit maturation. Because 'Bing', 'Chelan', and 'Skeena' have different timelines for the maturation of fruit after bloom, ethephon was applied at ca. 80% maturation for each of the cultivars in the 2014 growing season. This percentage coincided with 14 DBH for 'Bing', 12 DBH for 'Chelan' and 16 DBH for 'Skeena'

(Supplementary File 1). Information regarding ethephon treatment, and PFRF results for 2010 and 2013 can be found in Supplementary File 2.

PFRF measurements and abscission zone sampling

In all three years, sampling and measurements were conducted at the following time points for each sweet cherry cultivar: (1) immediately following application of ethephon or H_2O; (2) 6 hours after the application of ethephon or H_2O, and (3) at harvest.

At each sampling time, ten fruit were randomly selected for analysis from each of 4 trees/cultivar/treatment. PFRF was measured using a modified digital force gauge (Imada). In addition to the collection of PFRF values, the abscission zones of 10 fruit from each cultivar/treatment were harvested from corresponding trees at each time point per the following steps: 1.) Using a new razor blade, the fruit was first cut approximately 0.5 cm below the pedicel, leaving the pedicel and a thin disc of fruit/skin attached, 2.) two sets of parallel cuts were made downward on the cherry fruit disc on either side of the stem, effectively making a cubic piece of fruit 3mm x 3mm x 3mm attached to the pedicel, 3.) the pedicel was cut off directly above the fruit and the cube of fruit tissue consisting the abscission zone and some fruit and pedicel tissue was placed in a 15ml falcon tube and flash frozen for subsequent processing (Supplementary File 3).

Total RNA Extraction

Excised sweet cherry abscission zone tissue derived from 40 fruits / 4 trees / cultivar / time point was pulverized and homogenized into a single sample using a SPEX SamplePrep® FreezerMill 6870 (Metuchen, NJ USA) and the transferred to storage at -80°C. Total RNA was extracted using an acid guanidinium thiocyanate phenol chloroform extraction method similar to that previously described **(Chomczynski and Sacchi 1987)**. Briefly, 1mL of 0.8M guanidinium

thiocyanate, 0.4M ammonium thiocyanate, 0.1M sodium acetate pH 5.0, 5% w/v glycerol, and 38% v/v water saturated phenol were added to approximately 100 mg powdered tissue, shaken to evenly mix the sample and incubated at room temperature (RT) for 5 minutes. 200µL chloroform was added and shaken vigorously until the entire sample became homogenously cloudy and then was incubated (RT, 3 minutes). Samples were centrifuged at 17,000 x g at 4°C for 15 minutes, and the aqueous upper phase was transferred to a clean 1.5mL microcentrifuge tube. To this, 600µl 2-propanol was added, inverted 5-6 times, and incubated at RT for 10 minutes. Samples were centrifuged 17,000 x g at 4°C for 10 minutes, and the supernatant decanted. 1 mL 75% DEPC ethanol was added to the pellet, vortexed for 10 seconds, and centrifuged 9,500 x g at 4°C for 5 minutes. Pellets were then suspended in RNase free water and incubated at 37°C with RNase free *DNaseI* for 30 minutes, which was then inactivated at 65°C for 10 minutes.

Extracted RNA was quantified, and its quality was checked using the Bio-Rad (Hercules, CA) Experion system using the Experion RNA High Sensitivity Analysis kit or the Agilent (Santa Clara, CA) 2100 Bioanalyzer system using the RNA NanoChip and Plant RNA Nano Assay Class.

RNA sequencing and assembly

RNA samples that passed the quality threshold of RIN 8.0 were sequenced at the Michigan State University Genomics Service Center for library preparation and sequencing. The Illumina Hi Seq 2000 sequencing platform (San Diego, CA) was used to sequence 2x100 PE reads from the cDNA libraries generated from the above RNA extractions, representing each cultivar, treatment, and time point. cDNA and final sequencing library molecules were generated with Illumina's TruSeq RNA Sample Preparation v2 kit (San Diego, CA) and instructions with minor modifications. Modifications to the published protocol include a decrease in the mRNA

fragmentation incubation time from 8 minutes to 30 seconds to create the final library proper molecule size range. Additionally, Aline Biosciences' (Woburn, MA) DNA SizeSelector-I bead-based size selection system was utilized to target final library molecules for mean size of 450 base pairs. All libraries were then quantified on a Life Technologies (Carlsbad, CA) Qubit Fluorometer and qualified on an Agilent (Santa Clara, CA) 2100 Bioanalyzer.

Read preprocessing and assembly were conducted in CLC Genomics Workbench (8.5.1). Briefly, RNAseq read datasets were processed with the CLC Create Sequencing QC Report tool to assess read quality. The CLC Trim Sequence process was used to trim the first 16 bases due to GC ratio variability and for a Phred score of 30. All read datasets were trimmed of ambiguous bases. Illumina reads were then processed through the CLC Merge Overlapping Pairs tool, and all reads were *de novo* assembled to produce contiguous sequences (contigs). A single master assembly was generated from the combined read data from 'Bing', 'Chelan', and 'Skeena' cultivars at each time point (Supplementary File 4). Assembled contigs passed the filter criteria of >200 base length combined with >2x average read coverage. The cultivar-specific, non-trimmed read sets were mapped back to the master assembly to generate individual read mappings for each cultivar, treatment, and time point. Read counts were normalized for each mapping group using the Reads Per Kilobase per Million reads (RPKM) method (Mortazavi et al. 2008).

Differential expression analysis

A two-pronged approach, using Kal's Z-test and the NOIseq R package, was employed to identify contigs with highest likelihood of being differentially expressed (Kal et al. 1999, Tarazona et al. 2013). Only contigs that passed established threshold filtering for both methods were considered for further analysis.

Genes with highest probability of being differentially expressed were first identified using the NOISeq-sim package in OmicsBox, which is designed to infer probability of differential expression by modeling pseudo replications for RNAseq experiments in which sequencing replication is absent (in this case, due to pooling of biological replicates prior to RNA extraction) (Tarazona, Furió-Tarı, Ferrer and Conesa 2013, Tarazona et al. 2011). This approach has been successfully employed for differential expression analysis in other crops, including peach and rice (Altúzar-Molina et al. 2020, De La Fuente et al. 2015). Default parameters were used to simulate five replications with a set variability of 0.2 in each replication.

Next, Kal's Z-tests were performed in CLC Genomics Workbench 8.5.1 to add another level of stringency to the identification of differentially expressed genes. A paired experiment comparing the read count values for ethephon treatment to control values corresponding to each cultivar was performed.

The final sequence selection was reduced to 3,190 contigs, which were considered to have a high probability of being differentially expressed based on conformity to all of the following criteria for at least one treatment/time: (1) $|\log2FC|>1$, (2) NOIseq probability of DE > 0.8, (3) Kal's Z-test FDR corrected p-value <0.05 (Supplementary File 5).

Functional annotation and GO enrichment analysis

Assembled sweet cherry contigs were annotated using the Blast2GO feature in OmicsBox (version 1.2.4). Contigs were blasted for greatest sequence homology against the NCBI Viridiplantae database and subsequently assigned to their corresponding gene ontology (GO) terms as described previously (Götz et al. 2008, Hewitt, Ghogare, et al. 2020, Hewitt, Hendrickson, et al. 2020).

GO enrichment analysis using Fisher's Exact Test was also conducted in OmicsBox to identify cellular components, molecular functions, and biological processes that were over or under-represented in the ethephon treated fruit at harvest in comparison with the control fruit (Götz, García-Gómez, Terol, Williams, Nagaraj, Nueda, Robles, Talón, Dopazo and Conesa 2008). Based on the differential expression analysis, for each sweet cherry cultivar, lists representing transcripts with NOIseq probability>0.8, Kal's Z-test FDR corrected p-value<0.05, and logFC>0.5 at the harvest time point were produced. These lists served as the treatment datasets for enrichment analyses, and the master annotated transcriptome was used as the reference dataset. The FDR corrected p-value cutoff for enrichment was set to 0.05. Following separate enrichment analyses for each cultivar, enriched GO terms that were shared between cultivars or unique to a single cultivar were identified using the Venn Diagram application in OmicsBox.

qRT-PCR Validation

Targets for qRT-PCR validation were selected from a list of genes known to be involved in ethylene response and cell wall breakdown (Supplementary File 6). Primers were designed based on the near full-length transcript sequences to amplify an approximately 100-150 bp region in the 3' region of target transcripts. A bacterial luciferase gene was used as a spiked reference, with 50 ng added per reaction.

Library preparation, target amplification, and expression analysis were conducted in accordance with previously published methods, with minor modifications (Hendrickson et al. 2019). VILO cDNA synthesis kits (Invitrogen™) were used to generate three technical replicates of cDNA for each RNA isolation per manufacturer's instructions. Replicate cDNAs were then pooled into a single sample (50 ng/ul). qRT-PCR reactions were performed using iTAQ with ROX

and SYBR (BioRad) and 20µL reactions were prepared as per the manufacturer recommendations. A total of 2µL of cDNA diluted to 50ng/µL RNA equivalents was used per reaction with 5µL H_2O, 2µL of each primer (10µM), and 10µL of iTAQ SYBR® Green Supermix with ROX. The qRT-PCR reactions were performed on a Stratagene MX3005 using the following parameters: 95°C 5 min; 50 cycles of 95°C 30 sec, 57°C 30 sec, 72°C 30 sec; 72°C 5 min. Fluorescence readings were taken at the end of each elongation step. A melting step was performed following the cycles at 95°C for 30 sec, 54°C for 30 sec and ramp up to 95°C to produce a dissociation curve.

To capture PCR efficiency in the data, Cq values and efficiencies were calculated for each reaction using the LinRegPCR tool (Ramakers et al. 2003, Ruijter et al. 2009). Cq values resulting from efficiencies below 1.80 or above 2.20 were judged unacceptable and were treated as unsuccessful or undetected amplifications. Cq values with efficiency values that were within expected parameters but exceeded (or equaled) 40.00 were also deemed unacceptable and disregarded in downstream analysis. In the same manner, Cq values between (35.00-39.99) were determined to be of low confidence and were marked for special consideration in downstream analysis. Fold-change expression was determined from the Cq values of all gene targets (among all replicates of all samples) among the ‘Bing’, ‘Chelan’, and ‘Skeena’ cultivars using the Pfaffl method (Pfaffl 2001). Expression values were determined with reference to the luciferase spiked gene (Supplementary File 6).

Short variant identification

The GATK best practices pipeline for short variant discovery was used to identify SNPs and indels in key, differentially expressed ethylene- and auxin-associated contigs, with minor modifications (Poplin et al. 2018, Van der Auwera et al. 2013). Briefly, a group of paired,

untrimmed reads from each sweet cherry cultivar was aligned to a designated reference fasta file in CLC Genomics Workbench 8.5.1. This reference file contained only the sequences for contigs that had been previously assigned GO annotations in OmicsBox. The resulting three alignments were exported as BAM files for subsequent use in the GATK pipeline. A reference fasta index and dictionary were created using Samtools and Picard software programs, respectively. Within the GATK (v. 4.1.7.0) suite, the HaplotypeCaller tool was used to identify variants between parental haplotypes for each cultivar; the results from all three cultivars were then merged into a single GVCF file using the CombineGVCFs tool. Finally, The GenotypeGVCFs tool was used to perform joint genotyping on the GVCF file containing variant information for each cherry cultivar, and the results were visualized in Integrative Genomics Viewer (v. 2.8.2).

Results and Discussion

Pedicel-Fruit Retention Force

The PFRF values of control fruits decreased naturally over time, with reductions of 75.5%, 74.0%, and 37.5% observed for ‘Skeena’, ‘Bing’, and ‘Chelan,’ respectively. Application of ethephon at approximately 80% of final fruit size for the three cultivars decreased mean PFRF value in comparison with the respective controls, but whether this decrease was significant and resulted in the achievement of the threshold for mechanical harvesting varied across cultivars (Figure 1).

In ‘Skeena’, mean PFRF for both control and treatment fruit dropped below the 0.40kg-force (3.92N) threshold for mechanical harvesting, reaching final mean PFRF values of 0.29kg-force (2.84N) and 0.156kg-force (1.53N), respectively (Figure 1). These findings indicate that

while 'Skeena' is capable of natural abscission, ethephon application does significantly increase the AZ formation response, causing PFRF values to decrease significantly compared to control fruit by full fruit maturation.

Similarly, for 'Bing', mean PFRF for both control and treatment groups decreased over time; however, a mean PFRF conducive to mechanical harvesting was only achieved in the ethephon treated fruit, which reached a final PFRF value of 0.215kg-force (2.11N), significantly lower than the control value of 0.418kg-force (4.1N) (Figure 1). This suggests that the inducibility of 'Bing' is resultant of a similar, yet less dramatic, natural decrease in PFRF that is enhanced by ethephon treatment.

'Chelan' exhibited a statistically significant PFRF response at the time of harvest in the ethephon treated fruit in comparison with the control; however, the final PFRF of 0.832kg-force (8.16N) of the treatment group was not reduced to the threshold required for efficient mechanical harvesting (Figure 1). These physiological results support the observations that 'Chelan' forms neither a developmental nor an ethylene-induced PFAZ (Smith and Whiting 2010). However, given these findings and the trends of decreased PFRF observed in the treatment and control groups from each cultivar, there may be potential for induction of 'Chelan' abscission with the appropriate chemical targeting strategy aided by the gene expression results.

Transcriptome assembly and annotation

The transcriptome assembly resulted in the generation of 82,587 contigs from 1,061,563,488 total trimmed reads. Contigs were subsequently filtered for >200 base length and >2x coverage, for a final total of 81,852 contigs for downstream processing (Supplementary File

4). Functional annotation conducted using the OmicsBox genomics suite resulted in the assignation of annotations to a total of 30,946 (37.8%) contigs (Supplementary File 7).

Differential Expression of Ethylene-associated Contigs

The basis for the inducibility of PFAZ formation in 'Bing' during fruit development is evident at the genetic level with respect to expression of ethylene biosynthesis, signaling, and responsive elements in the transcriptome. Moreover, the results of short variant analysis lend a complementary insight regarding the basis for cultivar-specific differences in expression of ethylene-associated contigs.

At both the 0-hour time point and at harvest, in 'Bing', a transcript encoding the rate-limiting ethylene biosynthetic enzyme 1-aminocyclopropane-1-carboxylate oxidase 1 (ACO1) was highly elevated in expression, as was a transcript encoding ethylene receptor 2 (ETR2), a key gene involved in ethylene perception. In contrast, 'Skeena' and 'Chelan' displayed basal levels of expression of ACO1 and ETR2, suggestive of minimal ethylene response during abscission zone development (Figure 2).

Transmission of ethylene-activated signal to the nucleus involves ethylene-insensitive 2 (EIN2) and ethylene insensitive 2-like (EIL) family proteins. Upon activation of ETRs by ethylene, the C-terminal domain of EIN2 is cleaved and translocated to the nucleus where it activates transcription of ethylene-responsive factors (ERFs) involved in the regulation of downstream ripening responses (Liu et al. 2015; Chen et al. 2018). Numerous transcripts encoding ERFs were identified, which were differentially expressed in at least one cultivar and time point. Consistent with the aforementioned results, a majority of these contigs were highly induced in 'Bing' following ethephon treatment. In particular, contigs encoding ERF1B, ERF1B-like, and ERF109-

like were highly differentially expressed in 'Bing' 6 hours following ethephon treatment, and also at harvest; however, these genes did not change significantly in expression in 'Chelan' or 'Skeena' (Figure 3).

The dramatically heightened expression of ethylene biosynthesis, perception, signaling, and responsive genes in 'Bing' in comparison with 'Skeena' and 'Chelan' suggests the presence of cultivar-specific differences in either the coding sequences or in cis-regulatory components of ethylene-associated genes. Previously, a study investigating polymorphisms in the sweet cherry cultivar 'Stella' identified several missense mutations in ethylene biosynthetic ACS and ACO genes, and it was hypothesized that such mutations may alter the non-climacteric ripening profile with regards to ethylene response (Koepke et al., 2013). In this study, variant calling with GATK revealed several SNPs and indels in the ORFs and/or 5' UTR of contigs corresponding to ACO1 and ETR2, and EIN2 (Table 1). Interestingly, two SNPs unique to 'Bing' ACO1 correspond to non-synonymous mutations, imparting changes in the amino acid sequence and thereby likely affecting protein function (Table 1). Taken together with the expression results, these findings may explain the differential ethylene responses between cultivars, as well as the unique inducible abscission phenotype of 'Bing'.

Differential Expression of Auxin-associated Contigs

In addition to the differential ethylene responses, the ethephon-treated 'Chelan', 'Bing', and 'Skeena' PFAZ tissues displayed a differential reduction in the expression of auxin-responsive genes in comparison to their respective controls. This suggests that the response to auxin and/or reduction in auxin transport, was inhibited in the presence of exogenously applied ethylene.

Auxin-responsive transcription factors IAA11, IAA13, and IAA27, auxin transport facilitator WALLS ARE THIN 1 (WAT1), and Auxin Efflux Carrier (AEC) component 1c

displayed comparatively low expression in 'Skeena', intermediate expression in 'Bing', and higher expression in 'Chelan' control PFAZ tissues at harvest. Ethephon treatment attenuated this expression in PFAZ tissue for all three cultivars, but the same relative expression trend was maintained. WAT1, IAA13, IAA27, and IAA11 displayed decreased expression at harvest in all ethephon-treated cultivars in comparison with their respective controls. Interestingly, 'Chelan' displayed a natural increase in the expression of these transcripts from Day 0 to harvest, which was inhibited and reversed by the application of ethephon (Figure 4). For both 'Bing' and 'Skeena', the same transcripts naturally decreased in abundance over time—a decrease that was accelerated in the presence of ethephon.

Variant calling using the GATK pipeline revealed several SNPs and indels in the ORFs of IAA27, WAT1, and AEC 1b. In the case of IAA, a single base insertion at the end of the coding sequence in 'Bing' and 'Skeena' results in a change of the penultimate codon (and corresponding amino acid) and the premature introduction of a stop codon, leading to a one-residue truncation of the entire protein. Furthermore, in dAEC1b, a SNP was identified that imparts a single amino acid substitution only in 'Chelan'. In addition to the results of the expression analysis, these polymorphic regions within contigs corresponding to key auxin-associated genes may partially explain differential auxin responses among the three cultivars. Previously, it was demonstrated that the turnover of IAA/Aux family proteins is necessary for auxin response (Han and Hwang 2018, Reed 2001). Furthermore, the protein encoded by WAT1 is required for secondary cell wall formation as well as facilitation of vacuolar auxin transport, and increased expression of WAT1 has been shown to correlate with increased auxin mobilization and release of free IAA from vacuolar storage (Ranocha et al. 2010, Ranocha et al. 2011, Ranocha et al. 2013).

While free auxin was not measured in this study, differences in the abundance of auxin-responsive and auxin mobilization-associated transcripts in the three sweet cherry cultivars over time lends to extrapolation of information regarding cultivar-specific, endogenous free IAA concentrations. If free auxin levels are high, abundance and/or activity of auxin-responsive protein encoding transcripts (ARFs, IAAs) is expected to be higher to accommodate them. 'Skeena' does not require ethylene for abscission; however, ethephon application appeared to further offset the auxin to ethylene ratios, in favor of ethylene, to accelerate abscission in this cultivar. The ability of 'Skeena' to auto-abscise suggests that endogenous accumulation of free auxin at the site of abscission may be naturally lower in this cultivar than it is for 'Bing' and 'Chelan'. Conversely, the comparatively higher expression of transcripts associated with auxin response and movement proteins in 'Chelan' is suggestive of a naturally higher level of endogenous IAA. While the current industrial standard levels of ethephon application were insufficient to reduce the PFRF of 'Chelan' fruit to the threshold required for mechanical harvesting, both PFRF and auxin-associated transcript abundance in 'Chelan' did decrease as a result of ethephon application. It has been shown that ethephon application rates as high as 5.8 L ha^{-1} remain insufficient to induce a reduction of 'Chelan' PFRF values to the threshold for mechanical harvest (Smith and Whiting 2010). An early study found that a high application rate of 500 ppm (7.8 L ha^{-1} [6.7 pt A^{-1}]), ethephon begins to deleteriously affect some sweet cherry cultivars by inducing unwanted leaf abscission and terminal shoot necrosis, although it is not reported to what extent 'Chelan' is impacted by such a concentration (Bukovac et al. 1971, Smith and Whiting 2010). Considering the present results alongside the insight gained from previous work, it is possible that mechanical abscission of 'Chelan' could be achieved through a combination of a slightly higher ethephon application rate and application of auxin inhibitors, the latter of which could further shift the ethylene/auxin ratio

in a manner favorable to abscission while reducing the need for excessively high and potentially phytotoxic ethephon application rates.

'Bing' PFAZ tissues displayed an abundance of auxin-associated transcripts intermediate to that of 'Skeena' and 'Chelan'. Following ethephon treatment, the phenotypic observations of inducible abscission were supported at the gene expression level; with the responses greatly attenuated to lower than those of 'Skeena' ethephon treated and control PFAZ tissues.

qRT-PCR validation

qRT-PCR analysis of 10 ethylene-responsive/abscission-related genes resulted in 70% correspondence of general expression trends for 'Bing' and 'Chelan' and an 80% correspondence for 'Skeena'. Validated transcripts whose qRT-PCR expression pattern (fold change calculated using the $2^{-(\Delta\Delta Ct)}$ method) was consistent with that of the RNAseq-based expression (ethephon/control RPKM ratio) included genes associated with ethylene biosynthesis (ACS1 and ACO1), perception (ETR2), and response (ERF1B-like, ERF027-like, and WRKY1), as well as cell wall breakdown-associated polygalacturonase (PG) (Supplementary File 6).

Gene Ontology Enrichment Analysis

GO enrichment analysis, followed by filtering for most specific ontologies using the FDR-corrected p-value cutoff of 0.05, resulted in the identification of two enriched GOs for 'Skeena', seven for 'Bing', and 21 for 'Chelan'. Of these enriched terms, 15 were unique to 'Chelan', one was unique to 'Bing', and none were unique to 'Skeena'.

A single enriched GO term, 'integral component of membrane', was shared among all three cultivars. Differentially expressed genes associated with this term include cell wall and integrity-

444 associated patatin-like protein 2 (PLP2), which plays a role in programmed cell death, and WAT1,
445 which, in addition to its role in auxin mobilization, plays a role in secondary cell wall formation
446 and stability (Camera et al. 2009, Ranocha, Dima, Felten, Freydier, Hoffmann, Ljung, Lacombe,
447 Corratgé, Thibaud and Sundberg 2011). As all three cultivars undergo a decrease in PFRF
448 following application of ethephon, it is logical that biological processes, molecular functions, and
449 cellular components associated with membrane and cell wall integrity are affected (Figure 5).

450 A single GO term, 'response to stress', was shared between 'Skeena' and 'Chelan', but was
451 not enriched in 'Bing' (Figure 5). Based on this observation, it is plausible that 'Bing' has a lower
452 propensity to respond to and/or counteract the metabolically disruptive effects of ethephon (which
453 is expected to trigger ethylene-associated stress responses), and is therefore affected to a greater
454 degree than the other two cultivars, specifically with respect to induction of PFAZ formation.
455 Furthermore, it is possible that exogenous ethylene elicits a signal transduction response upstream
456 of abscission only in 'Bing', whereas the other cultivars may only perceive the exogenous ethylene
457 as environmental stress.

458 Interestingly, the GO term 'lateral root formation' was the only ontology that was uniquely
459 enriched for 'Bing' (Figure 5). One of the differentially expressed genes associated with this term
460 was the *lateral boundary domain containing protein 18* (*LOB18*), which has previously been
461 implicated in boundary formation during root development. It is possible that this gene, or
462 additional members of the LOB gene family, plays a role in boundary formation at the PFAZ
463 (Majer and Hochholdinger 2011). In addition to LOB18, a differentially expressed transcript
464 corresponding to receptor-like protein kinase HAESA-LIKE 1 (HSL1), is also associated with the
465 'lateral root formation' ontology, and mutation of HSL1 has was shown to impede floral and leaf

466 abscission in Arabidopsis (Niederhuth et al. 2013, Taylor and Walker 2018). The differential
467 expression of both LOB18 and HSL1 may underlie the ability to induce abscission in 'Bing'
468 through boundary formation and promotion of abscission-associated processes.

469 GO terms shared between 'Bing' and 'Chelan', but not enriched in 'Skeena', included
470 'auxin-activated signaling pathway', 'growth factor activity', 'plasma membrane', 'extracellular
471 region' and 'heme binding' (Figure 6). Consistent with the results of the expression analysis, it
472 appears that a greater degree of auxin response is present in these cultivars in comparison with
473 'Skeena'.

474 The most recalcitrant of the three cultivars to abscission zone formation, 'Chelan', had a
475 disproportionately high number of unique, enriched GO terms in comparison with the other
476 cultivars. A majority of these terms were associated with maintaining or regulating homeostasis
477 at the cellular, metabolic, and molecular levels, including: 'biological regulation', 'chemical
478 homeostasis', 'maintenance of location', 'maintenance of location in cell', 'regulation of gene
479 expression', 'regulation of cellular biosynthetic process', and 'regulation of biosynthetic process'.
480 Additional terms included: 'cellular response to auxin stimulus', 'cell-cell junction', 'cell
481 communication', 'binding', and 'plant organ development' (Figure 5). The high number and nature
482 of these unique, enriched terms is unlikely coincidental. 'Chelan' appears to have biological
483 networks in place for maintenance and regulation of inter- and intracellular homeostasis, which
484 may extend to the tissue or organ level, in the presence of external perturbation of the system by
485 ethephon. The overall nature of the enriched ontologies unique to 'Chelan' suggests that there is
486 also a high degree of coordination of cellular communication and response to stimuli, specifically
487 to auxin. The gene expression results lend insight into genetic and metabolic targets by which

'Chelan' and other non-abscising cultivars may be induced to abscise through appropriate chemical targeting of phytohormone pathways or, potentially, through genome editing of the identified ethylene- and auxin-associated genes involved in the abscission process.

Conclusions

This study investigated the changes in PFRF, gene expression changes, and implied biological processes underlying PFAZ formation in sweet cherry. The results provide a transcriptomic insight regarding the gene expression-level effects of exogenous ethylene application on the abscission phenotype and PFAZ development, which can be utilized by the industry to customize harvest strategies.

Consistent with previous work, PFAZ formation was observed to occur in a cultivar-specific manner, and the abscission phenotype of each cultivar was affected to a different degree by exogenous application of ethylene. Observation of unique, heightened expression of ethylene biosynthesis, perception, signaling, and response genes in 'Bing' following ethephon application and at harvest parallels the decrease in PFRF and may be partially responsible for the inducibility of abscission in this cultivar. Furthermore, the results of this study point towards a potential genetic basis for the inducible abscission response in 'Bing', with polymorphisms identified in key ethylene- and auxin-associated genes. The auto-abscission phenotype of 'Skeena' may result from naturally lower levels of free auxin, as evidenced by the low abundance of IAA/ARF-associated transcripts in comparison with 'Bing' and 'Chelan'. Furthermore, 'Skeena' exhibited fewer significant gene expression changes than the other cultivars following ethephon application, which corresponded to fewer enriched ontologies, suggesting that 'Skeena' may have a reduced capacity for homeostatic maintenance in light of perturbation by ethephon application. In 'Chelan', comparatively high abundance IAA/ARF-associated transcripts in control fruit, that was

attenuated following ethephon treatment, may indicate higher levels of endogenous free auxin. The increased capacity for auxin mobilization, indicated by IAA/ARF expression, antagonizes the abscission-promoting effects of exogenous ethylene. Furthermore, 'Chelan' exhibited the highest number of unique enriched gene ontologies, most of which were related to the maintenance of metabolic homeostasis. Taken together, these observations, along with the PFRF results, provide insight regarding the recalcitrance of 'Chelan' fruit to abscise at the pedicel-fruit junction.

Identification of cultivar-specific, differentially expressed genes and enriched GO terms involved in abscission, as well as ontologies that are shared across cultivars, provides information that will inform future efforts to promote controlled, timely abscission of sweet cherries. This, in turn, will lead to the improvement and standardization of mechanical harvesting, thereby improving efficiency and increasing the economic profitability of the sweet cherry industry. Ultimately, the outcomes of this work may be extended to other crops where planned abscission can be useful in managing the harvest.

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Author Contributions

SH – Transcriptome assembly, data analysis, manuscript preparation, and editing

BK – Experimental design, data analysis, manuscript preparation

TK – Experimental design, PFRF, and tissue collection

JA – Assisted with ethephon treatment and collection of PFRF data and tissue collection

MW – Experimental design, guidance on application of ethephon and tissue collection

AD – Conceived the study, experimental design, data analysis, manuscript preparation and editing.

Competing Interest

The authors declare no competing interests, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

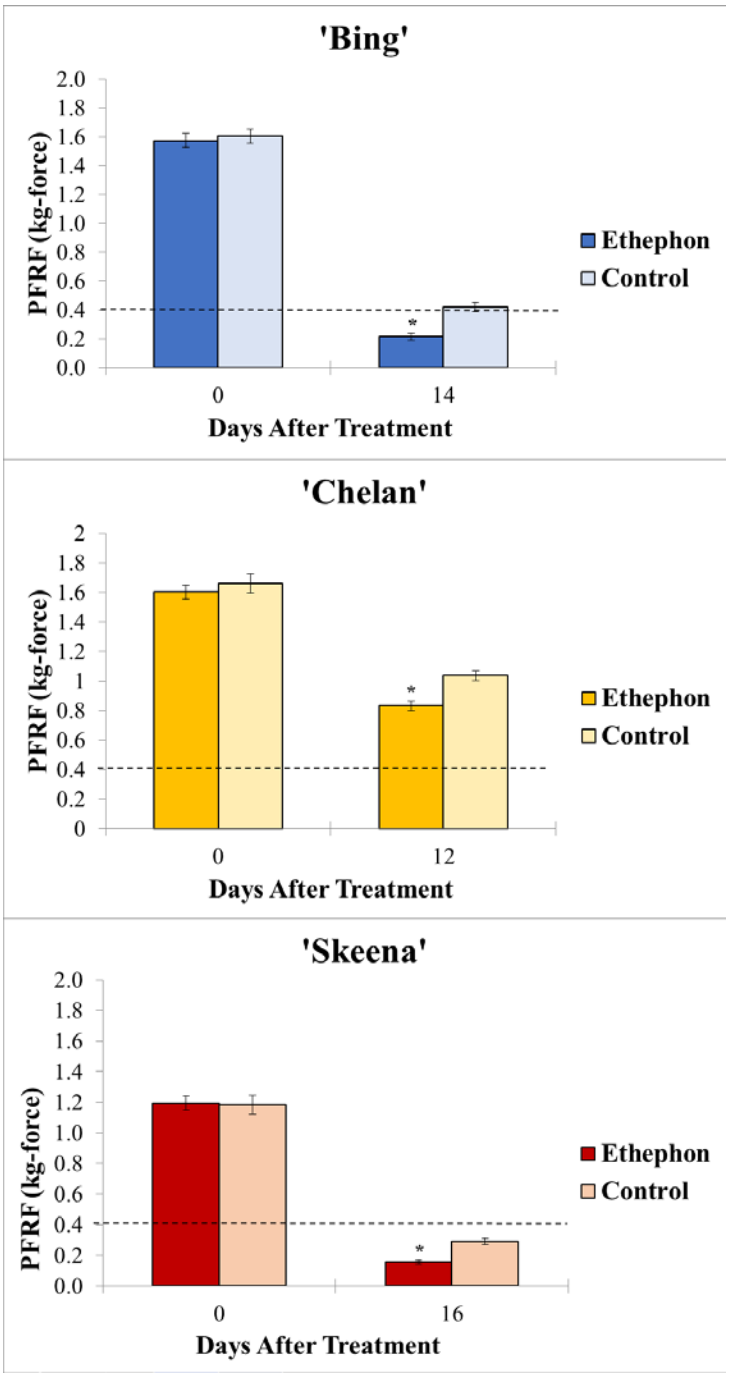

Figure 1. Endpoint mean control and ethephon-treatment PFRF values for (A) 'Bing' (B) 'Chelan', and (C) 'Skeena' PFAZ tissues. The dotted line represents the threshold PFRF for mechanical harvest. Asterisks indicate significant difference between ethephon-treated and control fruit at harvest ($p<0.05$).

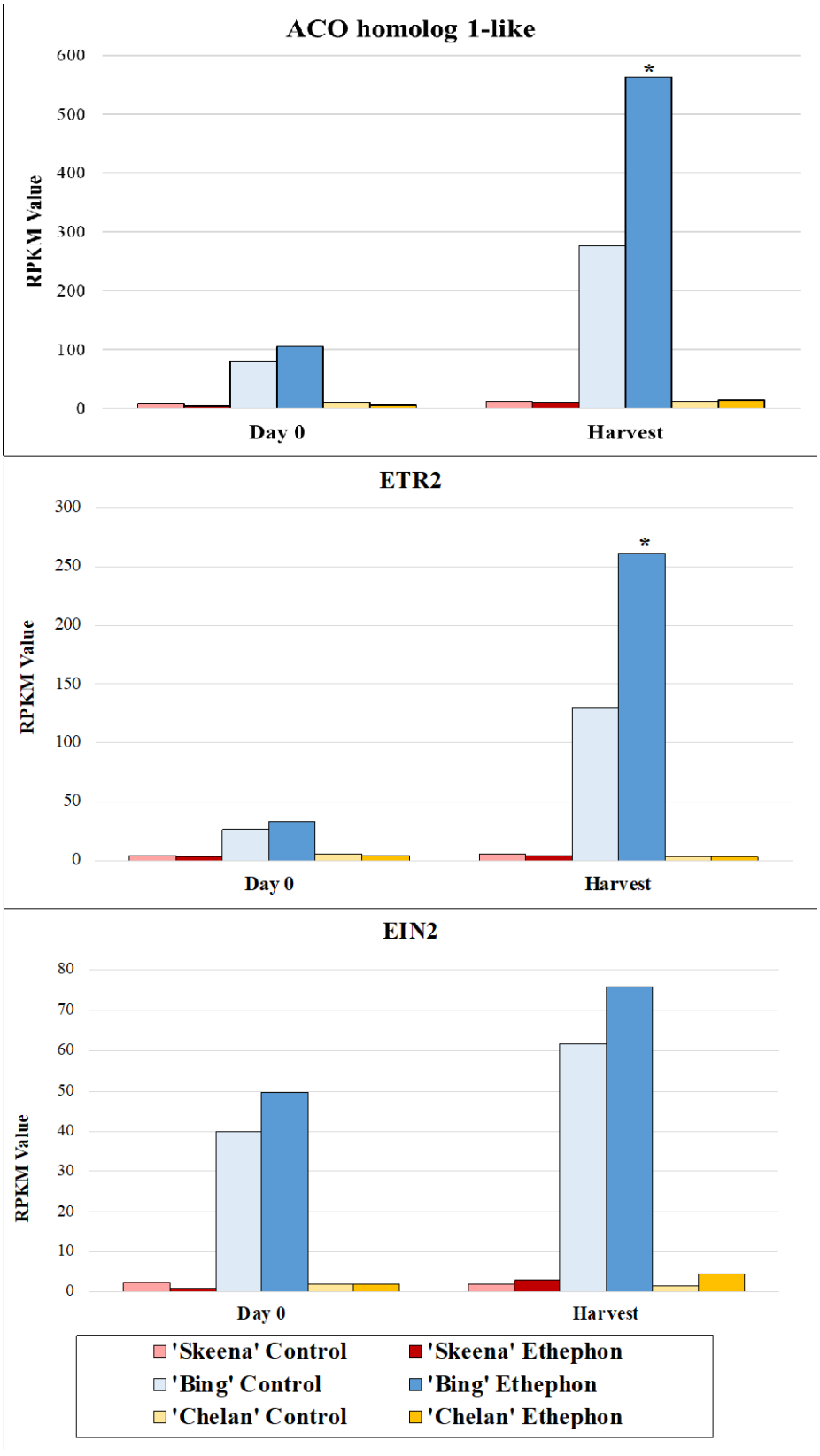

Figure 2. Expression patterns of ethylene biosynthesis gene 1-aminocyclopropane-1-carboxylate oxidase 1 (ACO1), ethylene receptor 2 (ETR2), and signal transducer ethylene insensitive 2 (EIN2) at Day 0 (0-hour) and at harvest. Asterisks indicate significant difference between ethephon-treated versus control fruit according to both Kal's Z-test and NOIseq differential expression probability analysis.

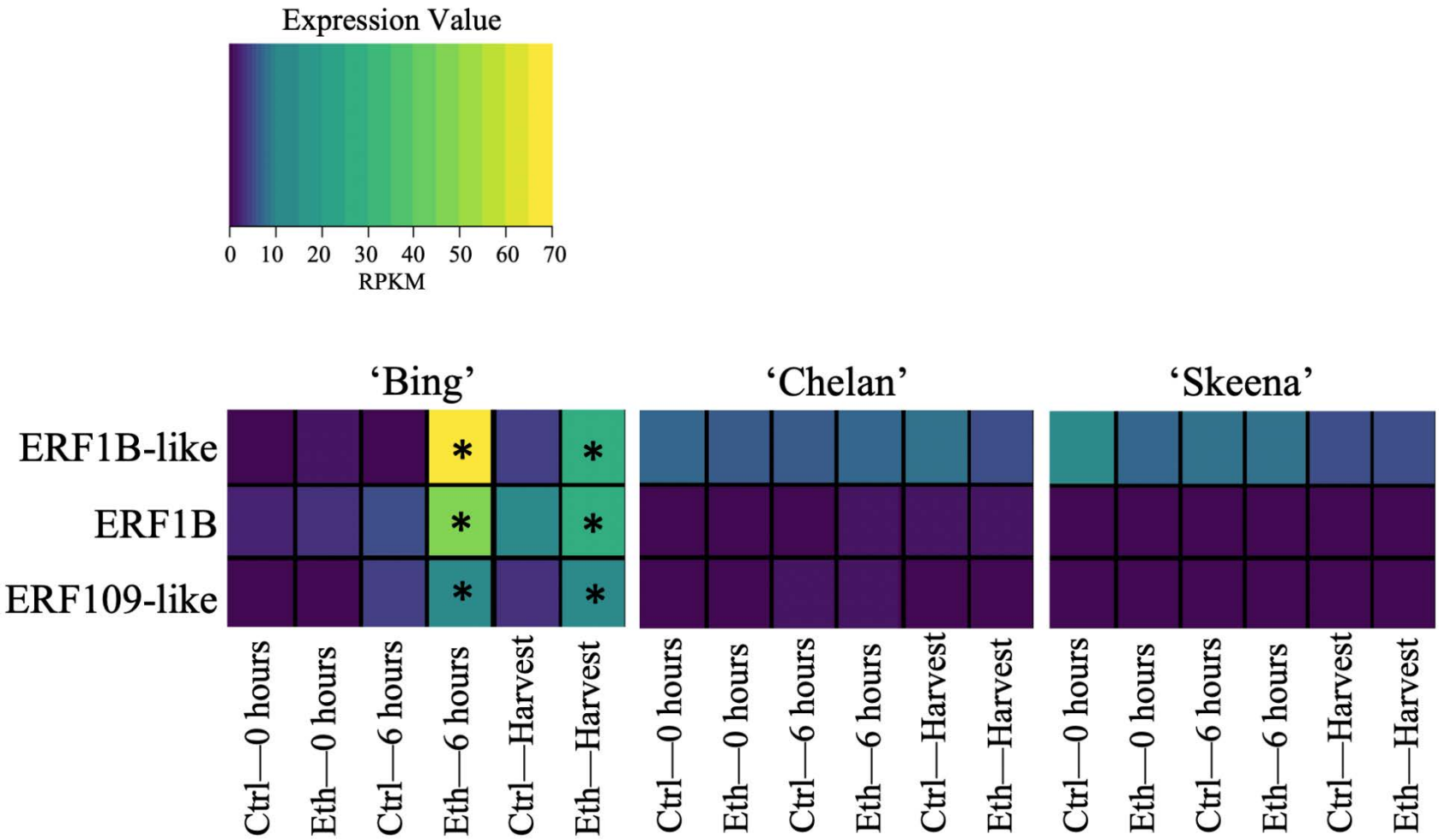

Figure 3. Heatmap displaying normalized expression values (RPKM) of three ethylene responsive transcription factor-encoding contigs displaying differential expression for at least one cultivar and timepoint. Expression was measured at 0 hours, 6 hours, and at harvest

for ‘Bing’, ‘Chelan’, and ‘Skeena’ cultivars. Asterisk indicates differential expression (Kal’s Z-test FDR-corrected p-value <0.05, NOISeq DE probability>0.8, $|LOG_2FC|>1$). Inducibility of ‘Bing’ abscission may lie in allelic difference in ERFs, as evidenced by cultivar-specific inducibility of expression ERF1B-like, ERF1B, and ERF109-like genes. Expression for ‘Skeena’ and ‘Chelan’ remained low, regardless of time point and treatment.

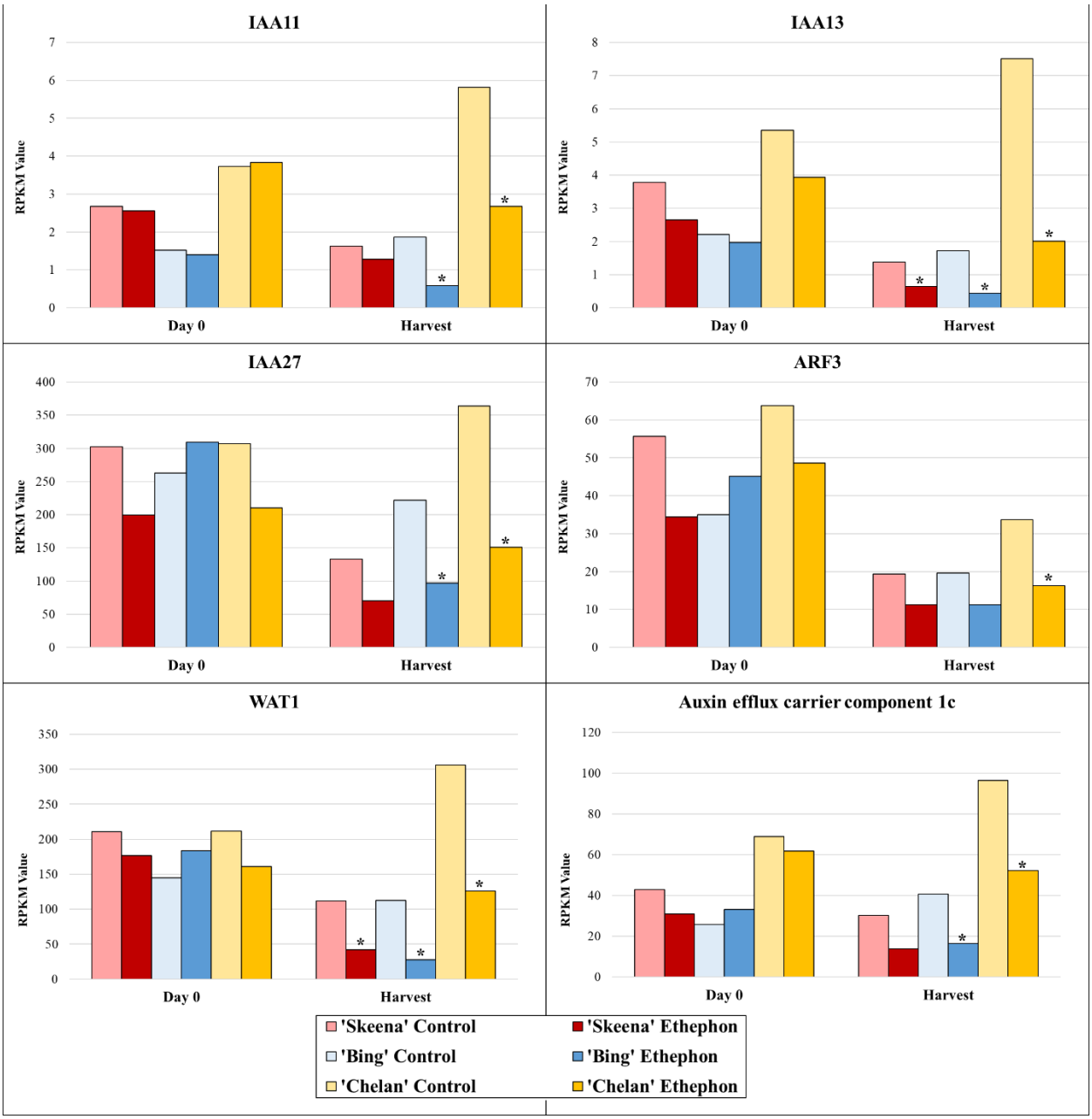

Figure 4. Expression patterns of auxin-associated genes at Day 0 (0-hour) and at harvest. Asterisks indicate significant difference according to both Kal's Z-test and NOIseq differential expression probability analysis. A shared expression pattern was observed for auxin-associated genes at harvest. Auxin responsive transcription factors IAA11, IAA13, and IAA27, auxin transport facilitator WALLS ARE THIN 1 (WAT1), and auxin efflux carrier component 1c displayed

comparatively lower expression in ‘Skeena’, intermediate expression in ‘Bing’, and higher expression in ‘Chelan’ control at harvest. Following ethephon treatment, expression was attenuated in all three cultivars, however the same relative expression trend was maintained, with ‘Skeena’ ethephon-treated fruit exhibiting lowest expression at harvest and ‘Chelan’ ethephon-treated fruit displaying the highest expression at harvest.

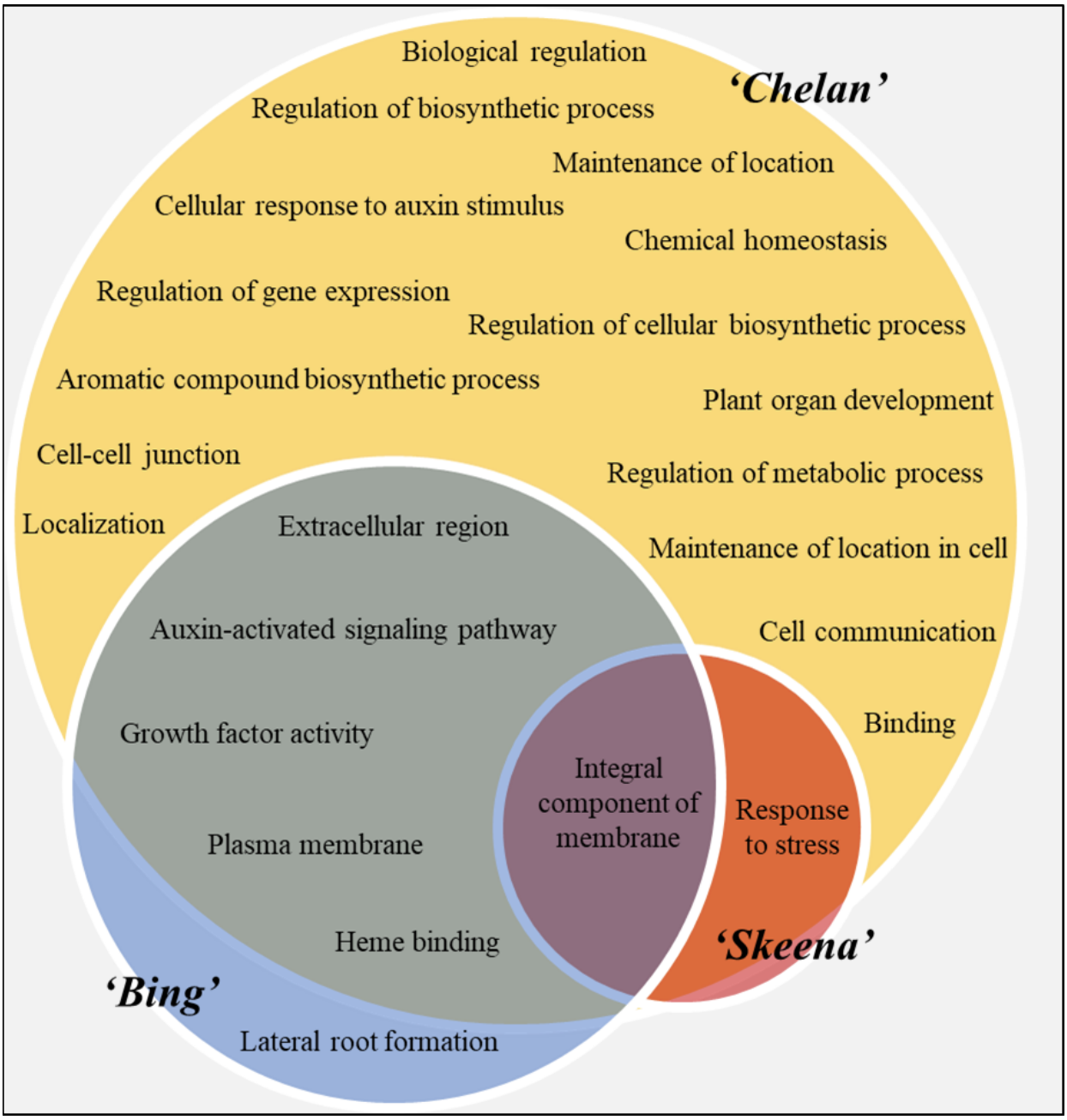

Figure 5. Shared and unique enriched GO terms in ethephon-treated 'Bing', 'Chelan', and 'Skeena' fruit at harvest versus control fruit at harvest. Enrichment results are based on Fisher's Exact test with an FDR corrected p-value <0.05.

Table 1. Variants identified in key, differentially expressed ethylene and auxin-associated genes. The GATK RNAseq short variant discovery pipeline was used for characterization of SNPs and indels (Poplin, Ruano-Rubio, DePristo, Fennell, Carneiro, Van der Auwera, Kling, Gauthier, Levy-Moonshine, Roazen, Shakir, Thibault, Chandran, Whelan, Lek, Gabriel, Daly, Neale, MacArthur and Banks 2018).

Gene	Translated Protein Length (residues)	Variant Residue Number (CDS) or Sequence Location (UTR)	Variant Type	Amino Acid/ ORF change	Genotype		
					Bing	Chelan	Skeena
ACO1 Contig 5131 (1562 bp)	376	114 63 49 5' UTR (-15 bp) 5' UTR (-99 bp)	SNP, A-->T SNP, C-->T SNP, A-->G SNP, A-->G SNP, T-->C	Val-->Asp None, Synonymous Leu Ser-->Pro N/A N/A	A/T C/T A/G A/G T/C	A/A C/C A/A A/A T/C	A/A C/C A/A A/A T/T
ETR2 Contig 6010 (3113 bp)	764	5' UTR (-331 bp)	Indel, T-->TA	N/A	T/T	T/TA	T/TA
EIN2 Contig 2549 (2771 bp)	576	5' UTR (-99 bp)	SNP, C-->A	N/A	C/A	C/C	C/A
IAA27 Contig 864 (2374 bp)	319	318 144	Indel, C-->CT SNP, T-->C	Ser-Asn-Stop-->Lys-Stop None, Synonymous Ala	C/CT T/C	C/C T/C	C/CT T/T
WAT1 Contig 1306 (1902 bp)	406	3' UTR (+75 bp) 324 241	Indel, C-->CT SNP, G-->C SNP, C-->T	N/A None, Synonymous Val None, Synonymous Lys	C/CT G/C C/T	C/C G/G C/C	C/C G/G C/T
Auxin Efflux Carrier Contig 2503 (1568 bp)	249	95 142	SNP, A-->G SNP, C--->A	Asp-->His None, Synonymous Gly	A/A C/C	A/G C/A	A/A C/C

Supplementary File 1. Ethephon treatment and sampling timeline

Supplementary File 2. PFAZ data from 2010, 2013, and 2014 seasons

Supplementary File 3. Picture of fruit section sampled for PFAZ analysis

Supplementary File 4. Cherry assembly fasta file

Supplementary File 5. Spreadsheet with RPKM values of contigs that passed stringent statistical and probability filters for differential expression based on Kal's Z-test and NOIseq analysis.

Supplementary File 6. qRT-PCR primers; Cq values and fold change expression summary by template

Supplementary File 7. List of all annotated contigs >2x coverage and >200bp in length

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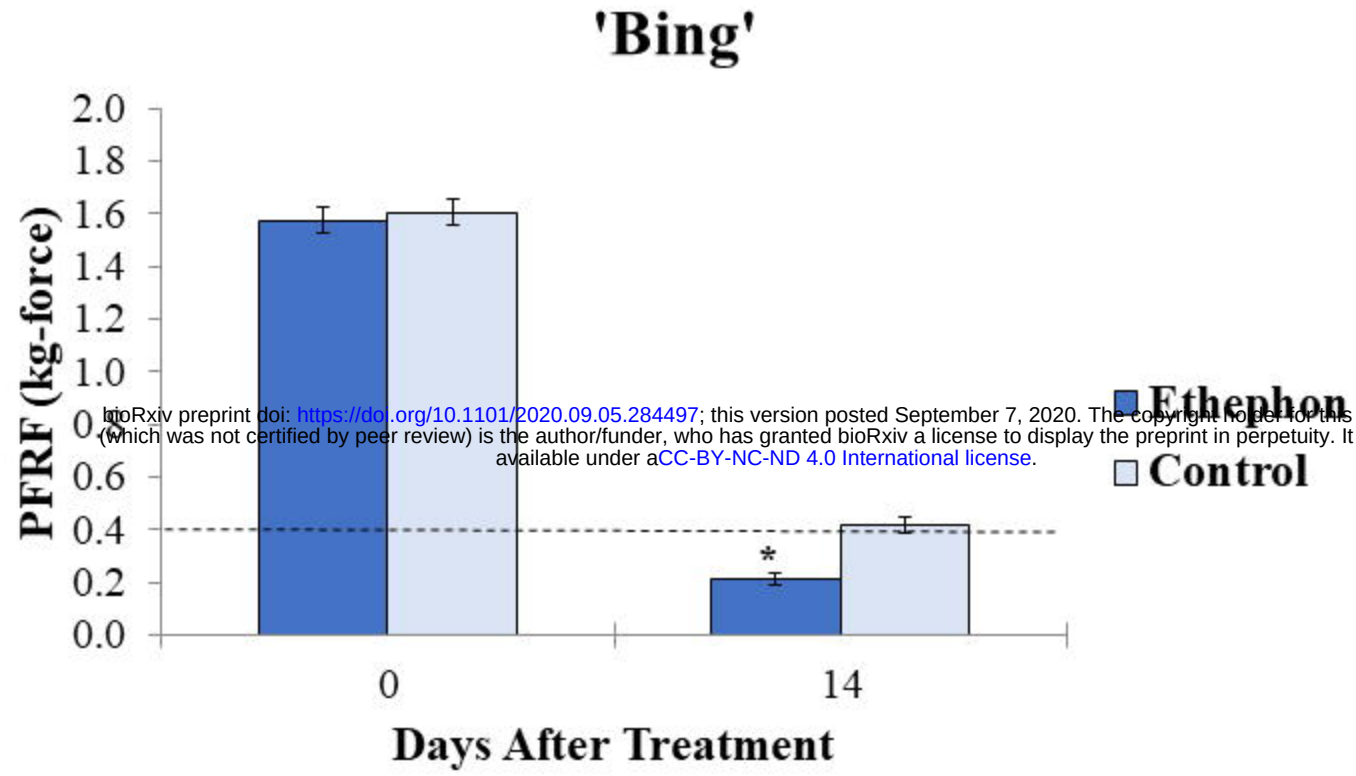

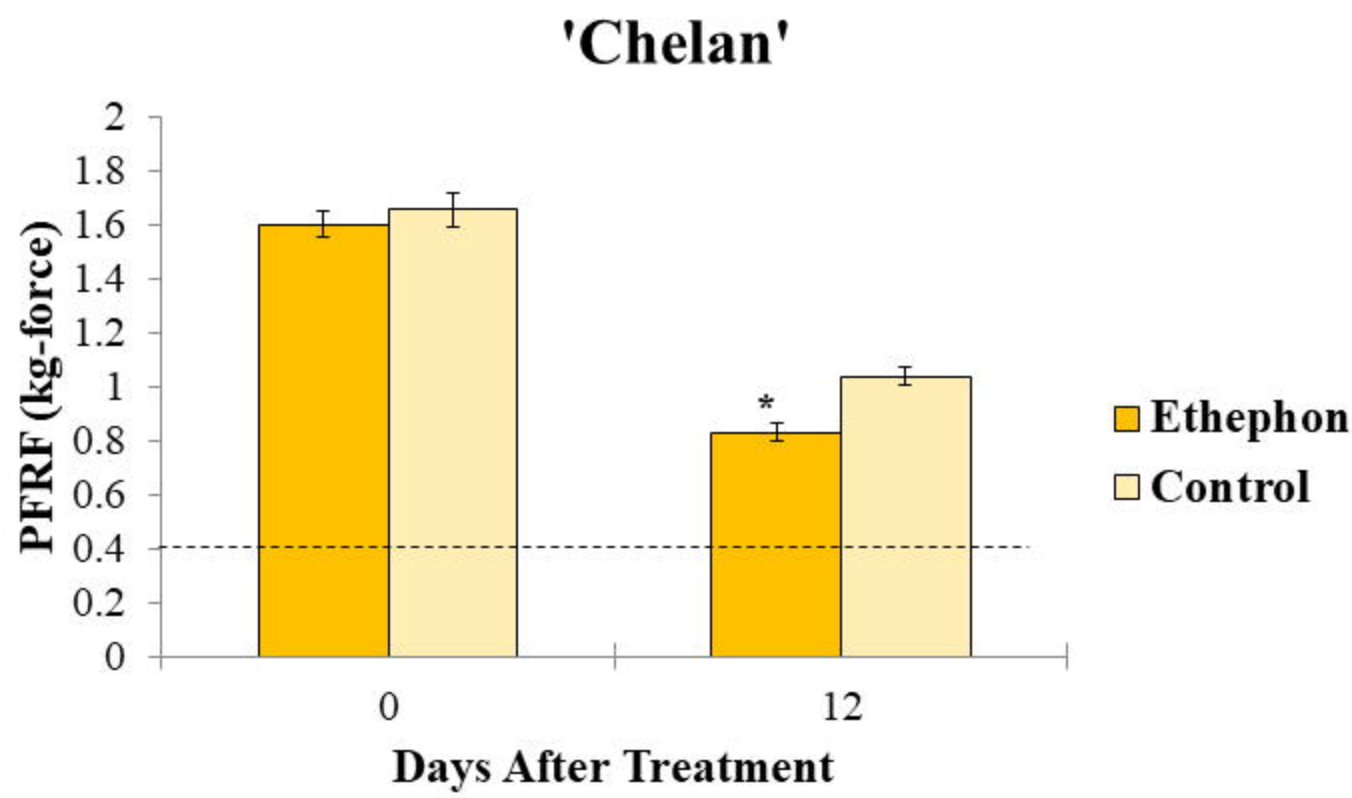

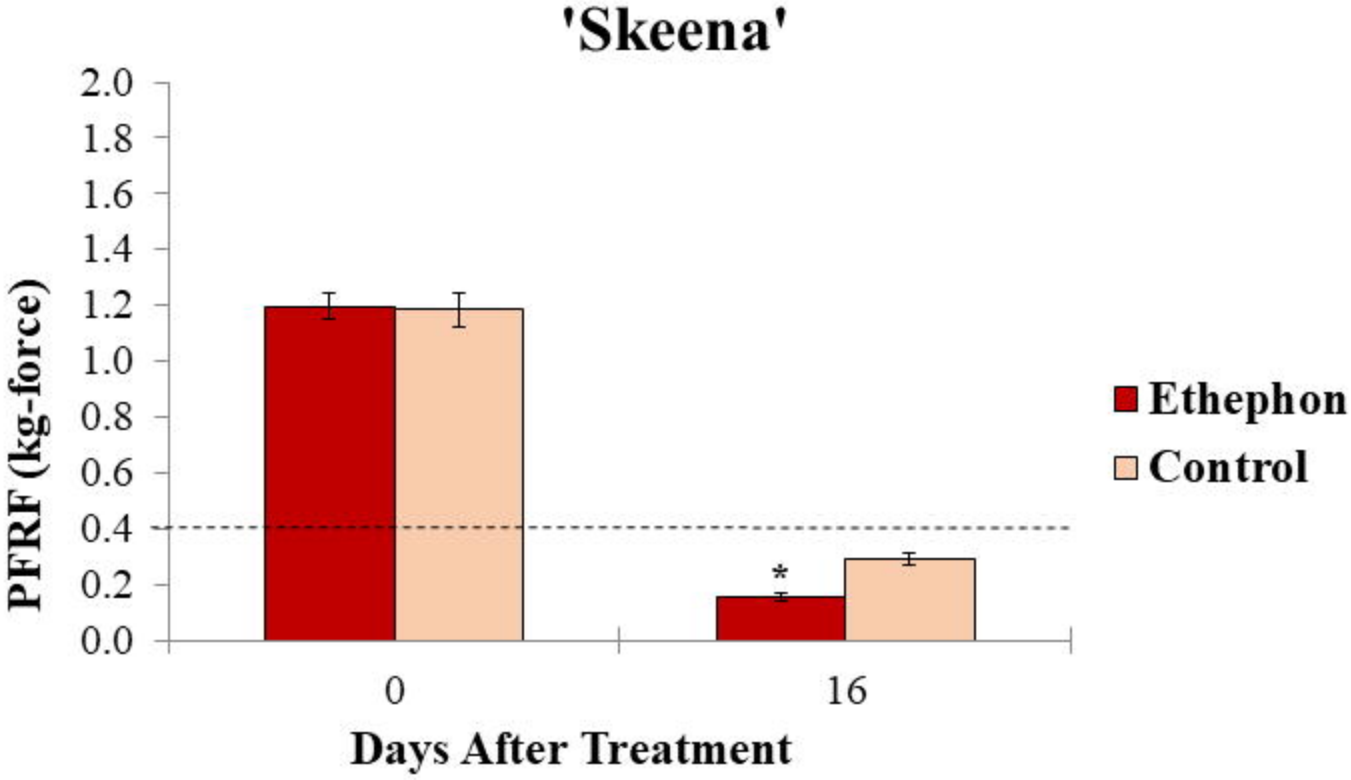

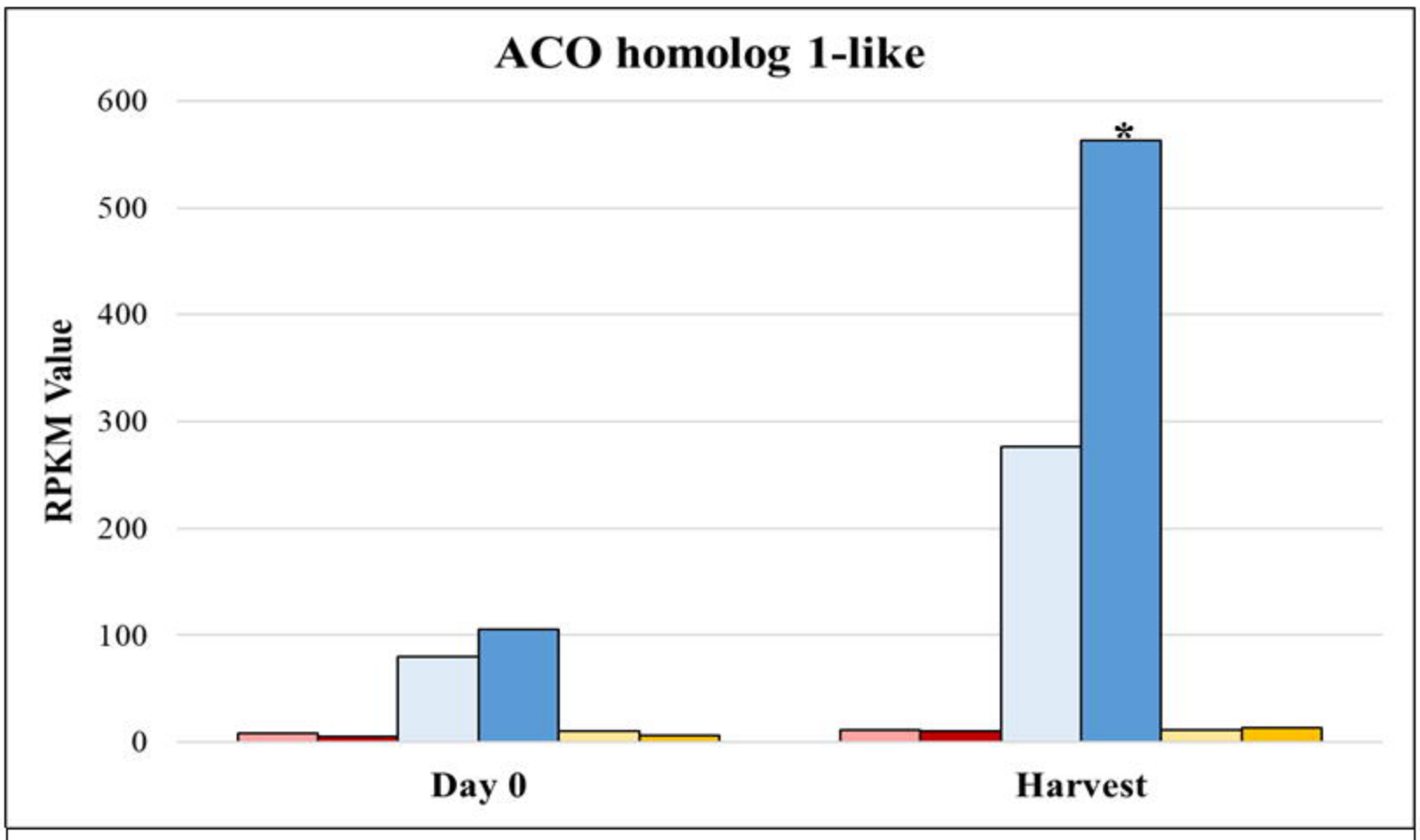

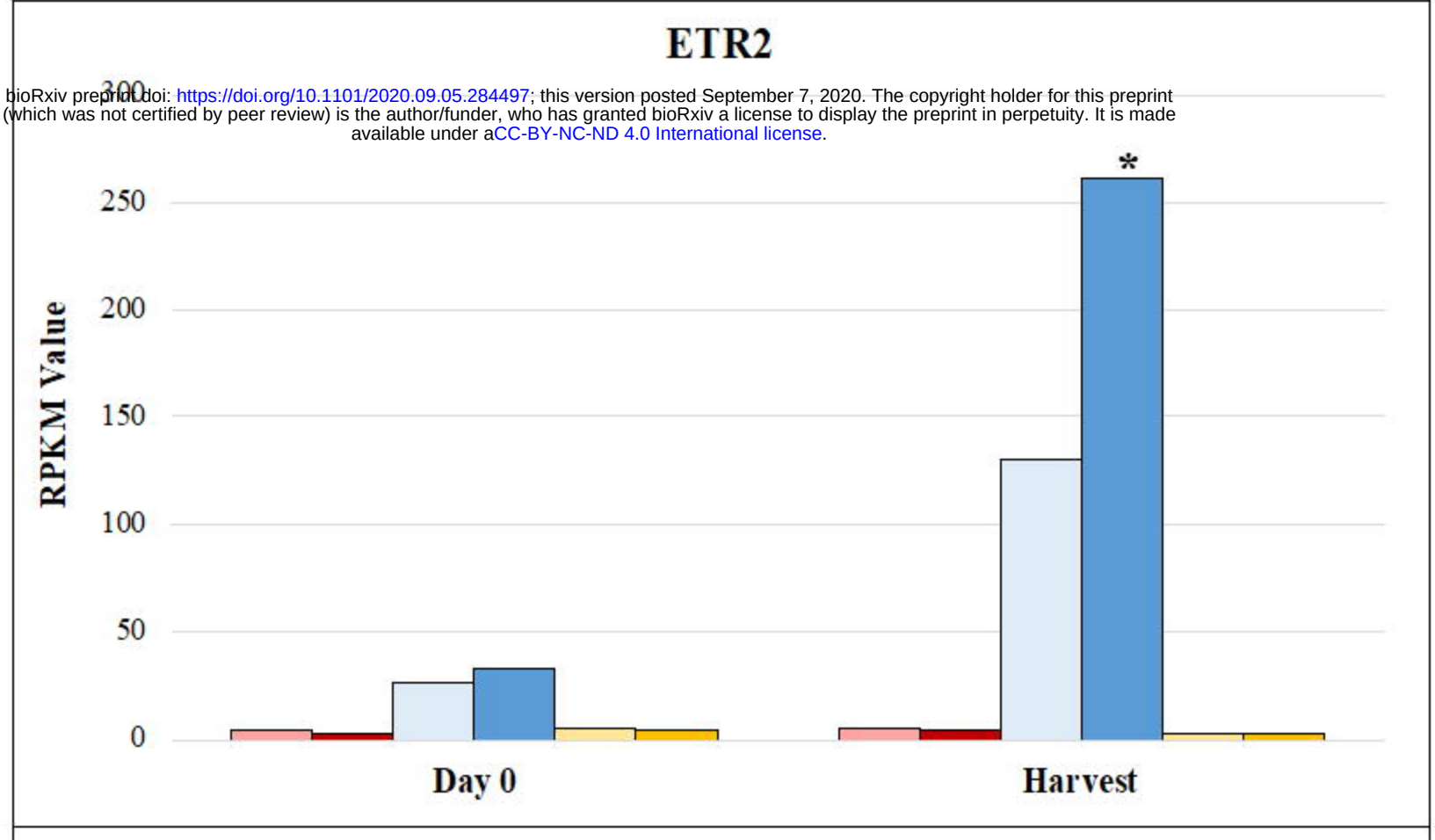

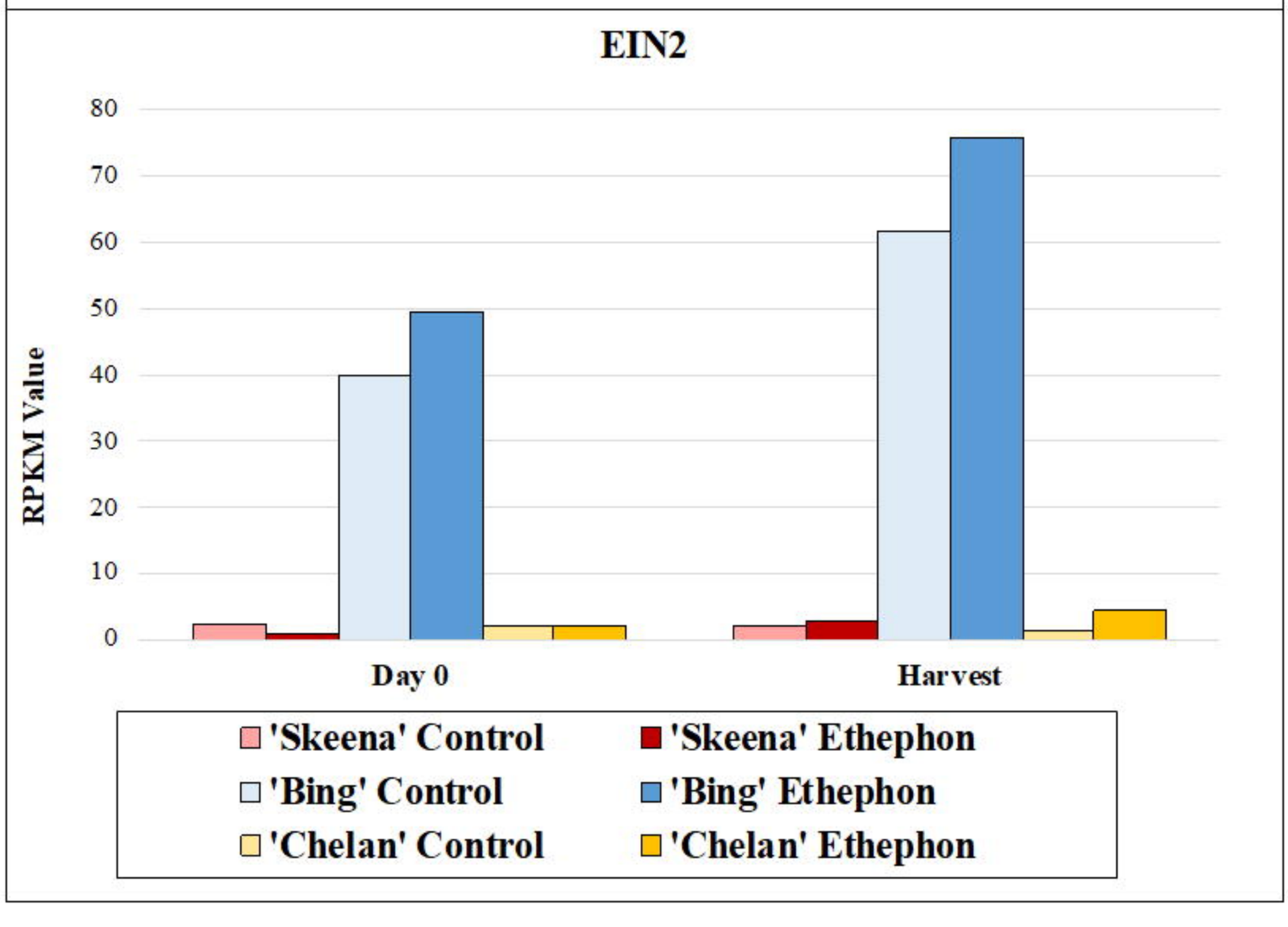

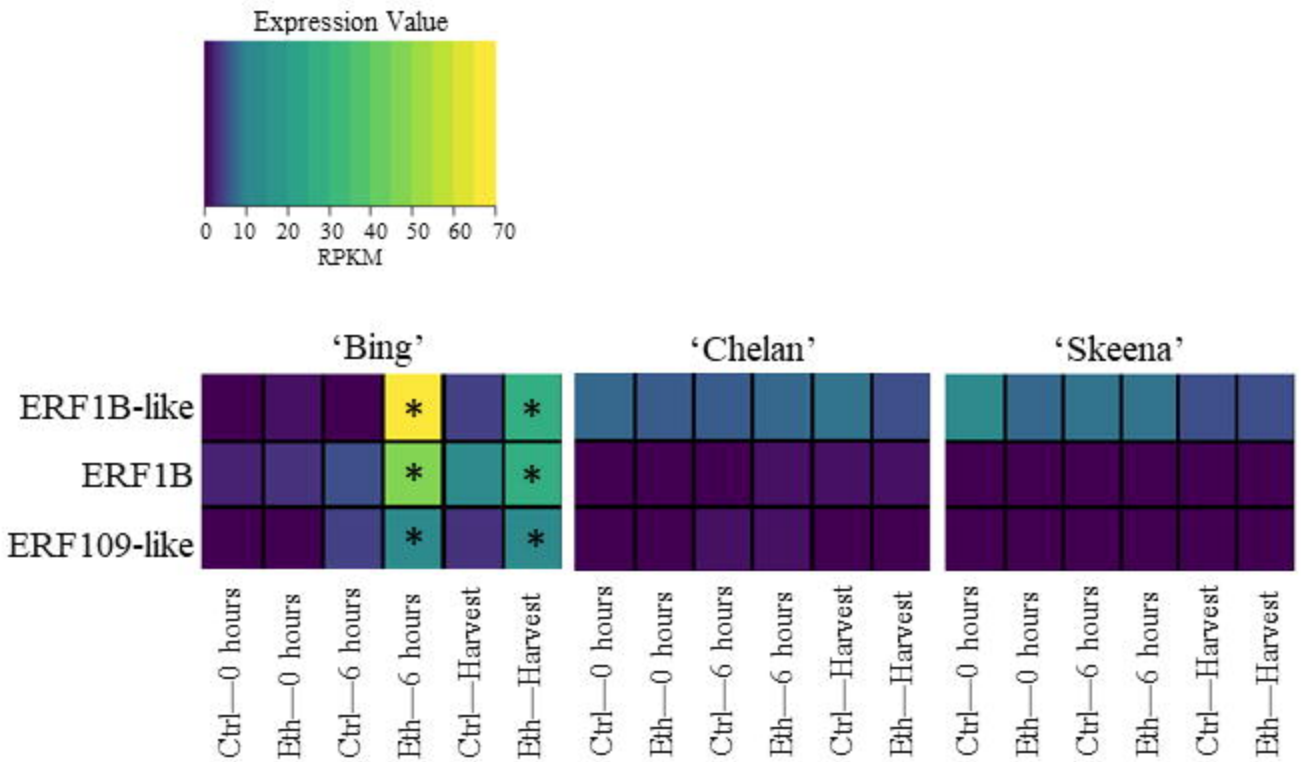
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'Chelan'
'Skeena'
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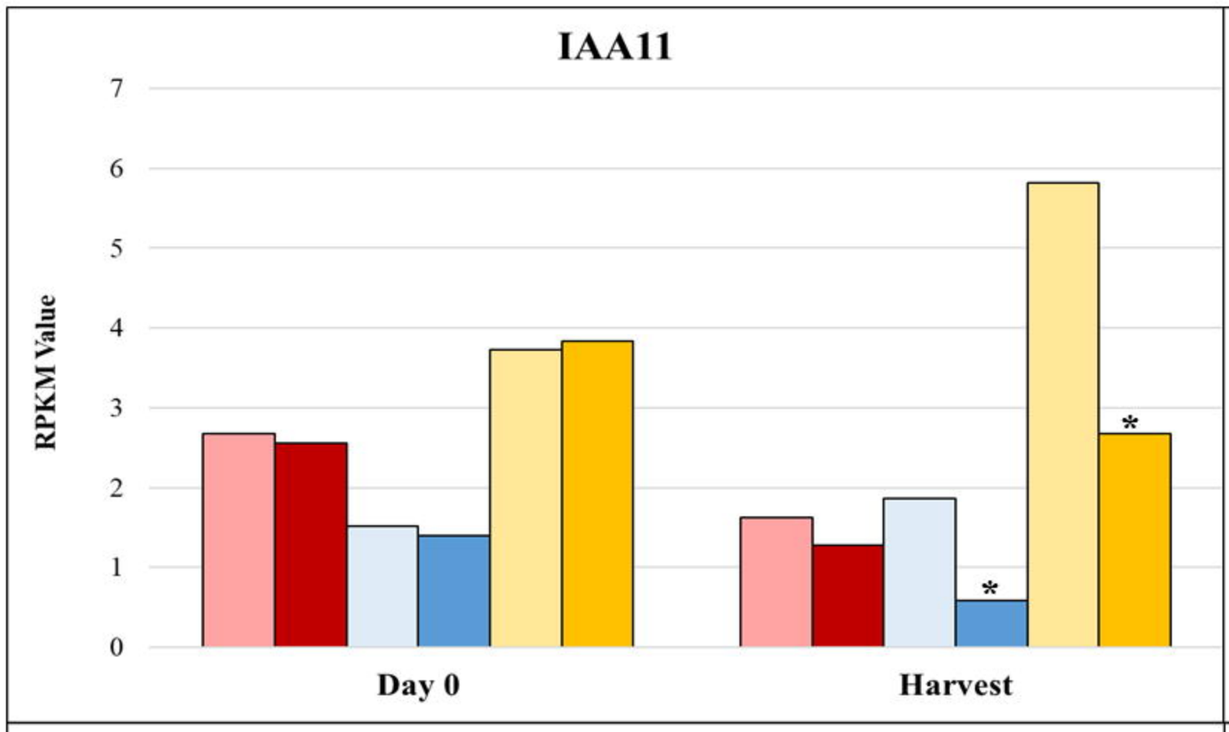
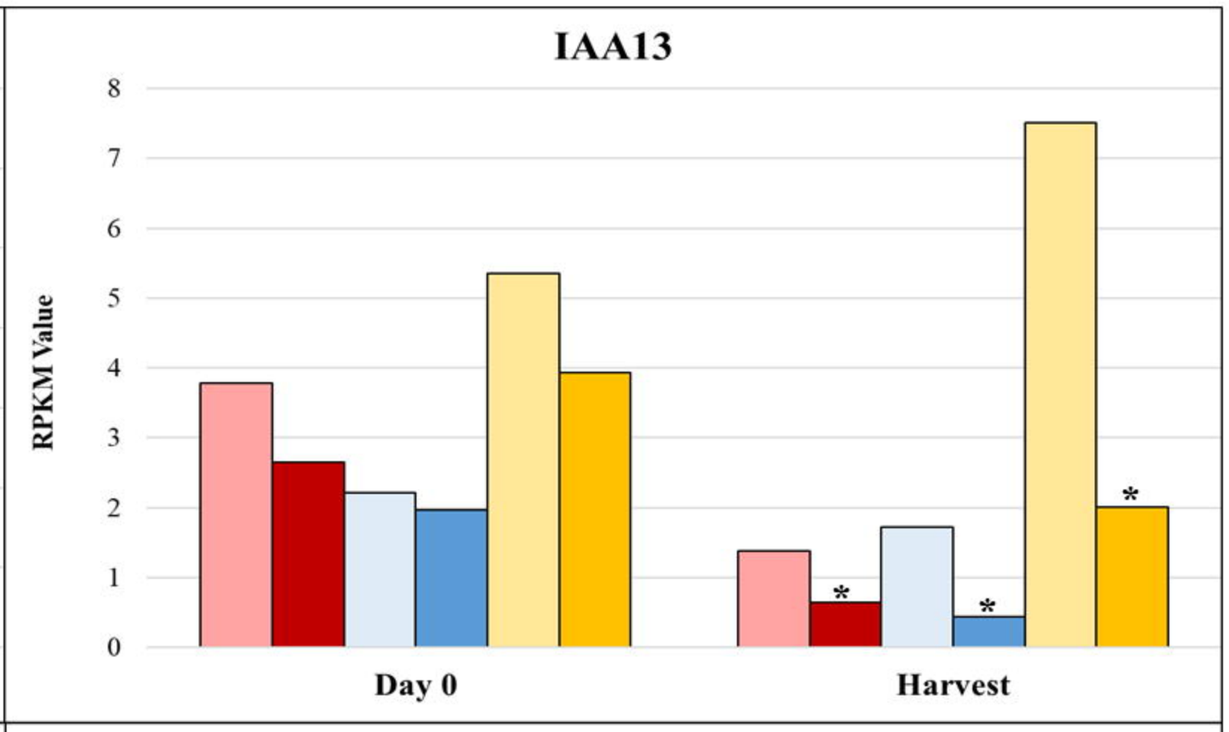
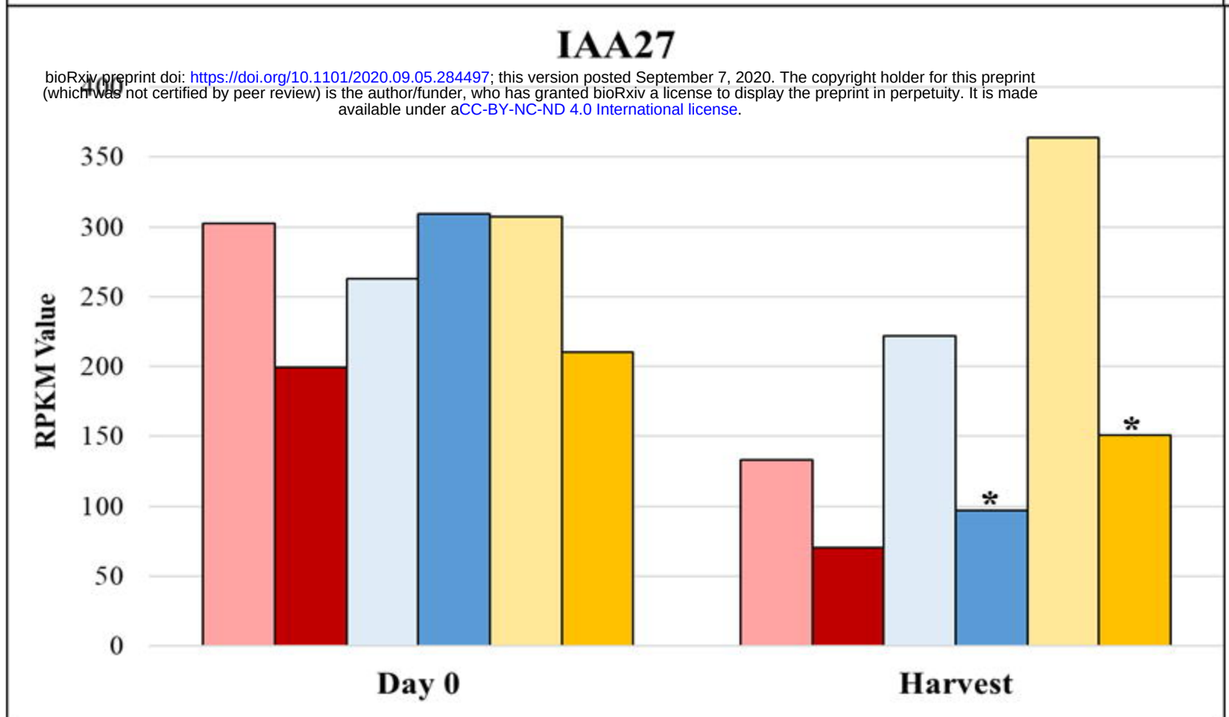
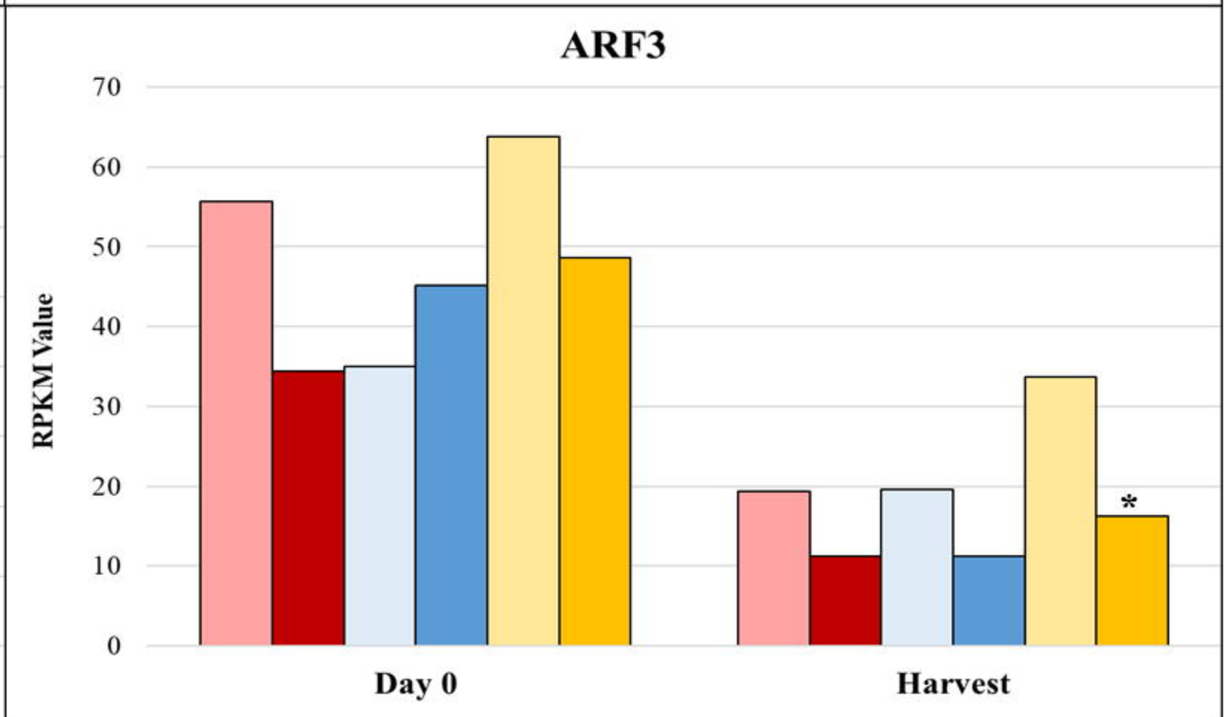
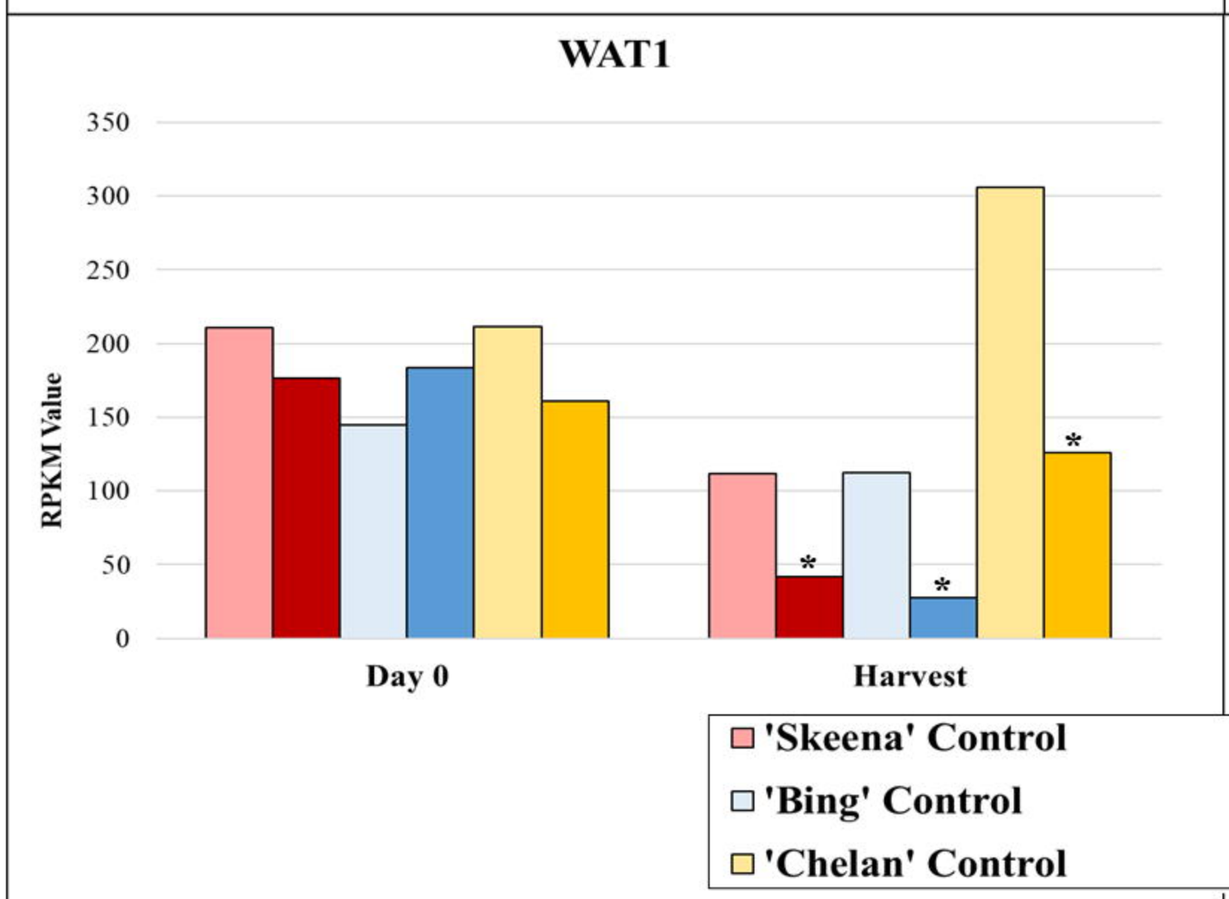
Ctrl—0 hours
Eth—0 hours
Ctrl—6 hours
Eth—6 hours
Ctrl—Harvest
Eth—Harvest

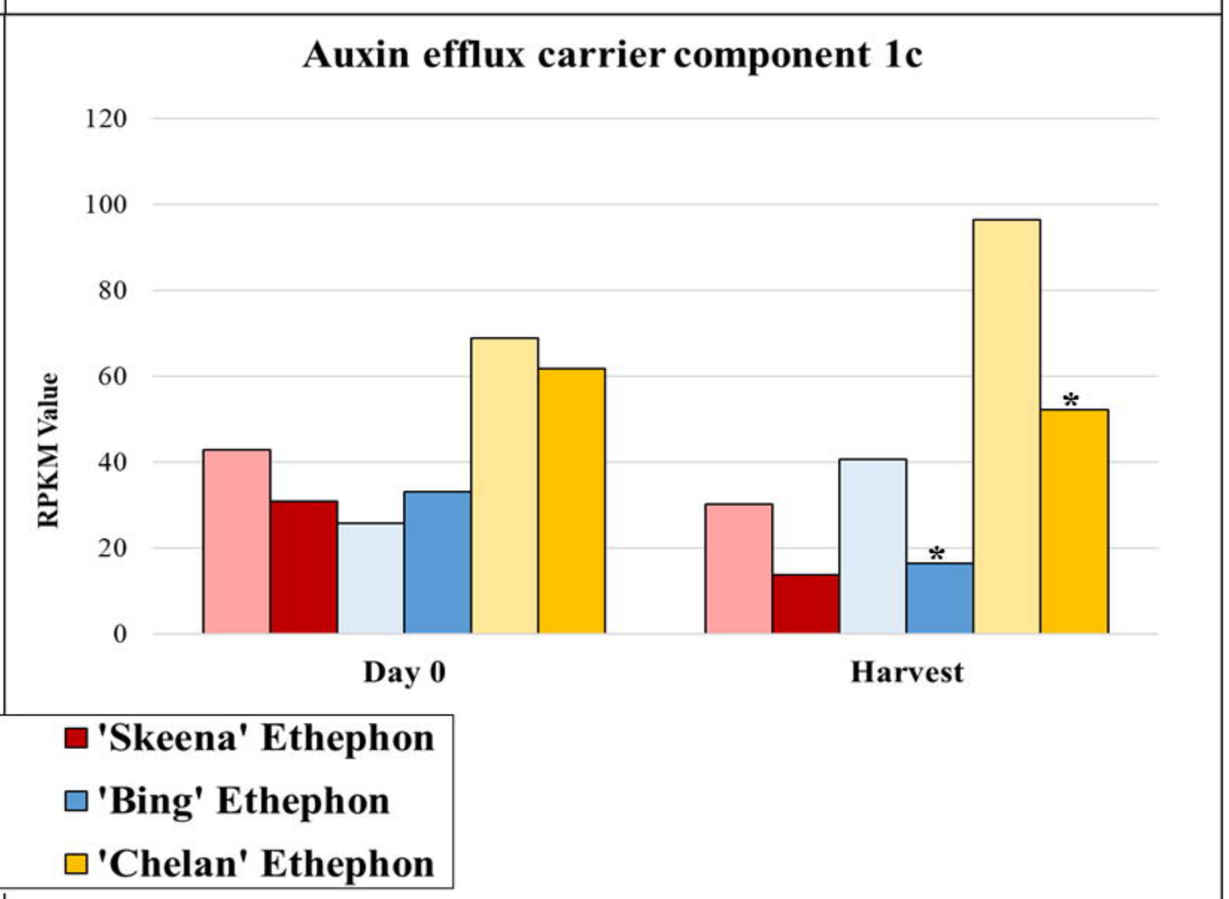


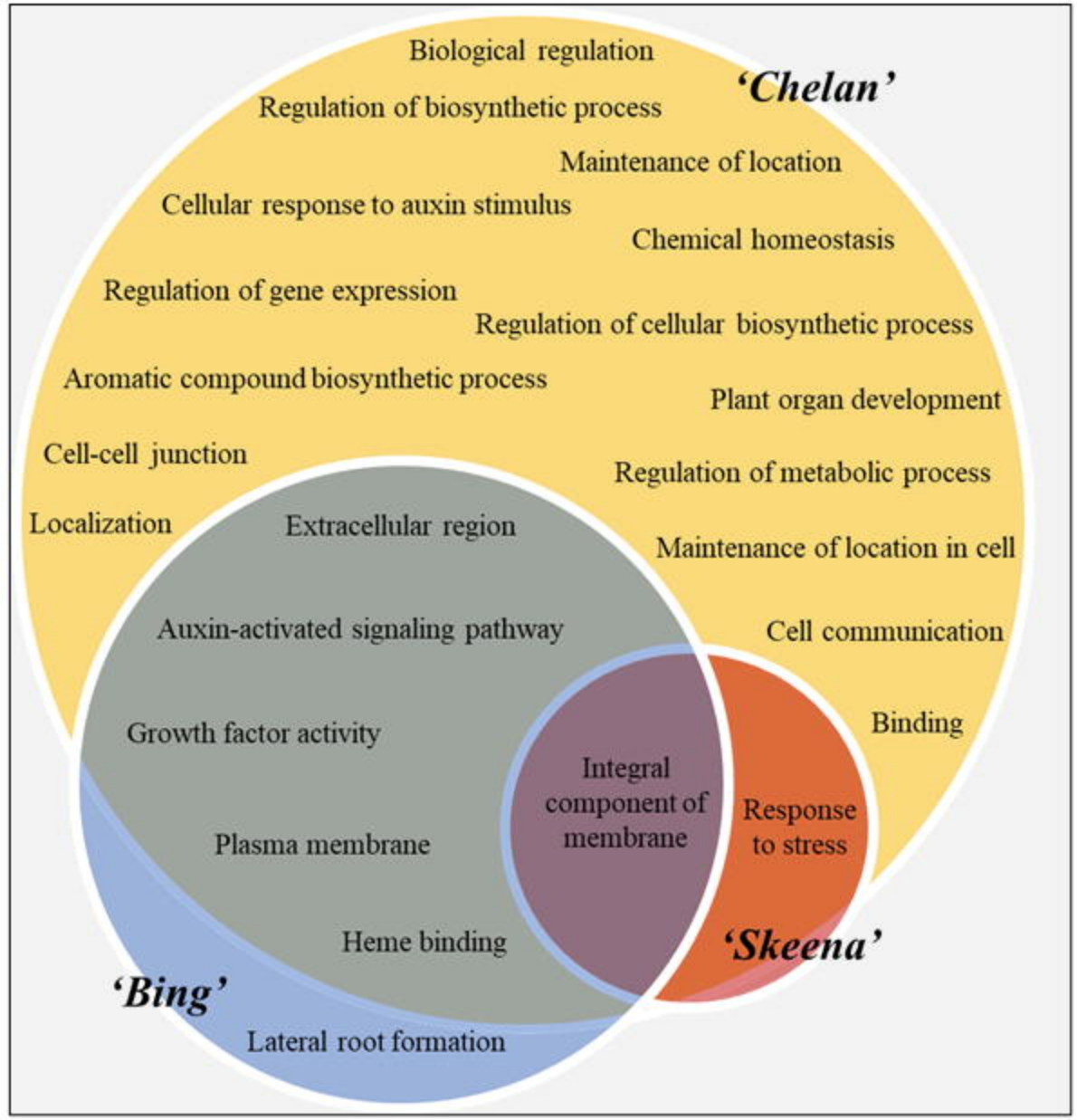











Biological regulation
'Chelan'
Regulation of biosynthetic process
Maintenance of location
Cellular response to auxin stimulus
Chemical homeostasis
Regulation of gene expression
Regulation of cellular biosynthetic process
Aromatic compound biosynthetic process
Plant organ development
Cell-cell junction
Regulation of metabolic process
Localization
Extracellular region
Maintenance of location in cell
Auxin-activated signaling pathway
Cell communication
Growth factor activity
Binding
Integral component of membrane
Response to stress
Plasma membrane
Heme binding
'Skeena'
'Bing'
Lateral root formation